# THE WILMS' TUMOUR GENE, *WT1*, IN THE DEVELOPMENT OF THE MOUSE EMBRYO AND ITS KIDNEY

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## DECLARATION

I declare

(a) that this thesis was composed by myself, and

(b) that the work presented is my own, except where stated.

Jane Frances Armstrong

## ABSTRACT

Wilms' tumour (WT), a renal tumour of early childhood, arises as a result of uncontrolled proliferation in the stem cells of the kidney due to an anomaly in their developmental pathway. A strong genetic element is known to be involved in its genesis, with more than one gene being implicated. The first of these, *WT1*, has recently been isolated and shown to be expressed in a specific pattern in both the developing human kidney and the tumour itself. The purpose of the work presented in this thesis was to examine the role that this gene plays in mouse embryogenesis, a system that lends itself to experimentation more readily than the human. Here, the expression of the mouse homologue of *WT1* was examined in three complimentary systems; the developing mouse embryo, the kidney as it formed both *in vivo* and *in vitro* and finally, in a possible mouse model for WT.

A comprehensive study of the expression pattern of *WT1* during development was undertaken, using *in situ* mRNA hybridisation. Expression was first apparent in a small area of the lateral mesoderm in the 9 day embryo. Within 12 hours this had increased markedly, with both the lining of the whole coelomic cavity and the early urogenital ridge being labelled. As development proceeded, expression was initiated in a limited set of tissues which included the metanephros, the mesothelium, the gonads, the spleen, the developing body-wall musculature, the spinal cord and the brain. Expression was present in 15 day embryos but markedly reduced by 19 days with labelling being restricted to the kidney.

The expression of *WT1* in cultured kidney rudiments was then examined and found to be consistent with that observed *in vivo*. The gene was expressed at a low level in condensed mesenchyme, with a much higher level being detected in the developing renal corpuscle. Using the transfilter system of organ culture, it was shown that *WT1* was expressed in the cap of condensed metanephric mesenchyme prior to induction and that this level increased after induction, a result confirmed using very early 11 day embryos.

The mouse model of WT involved placing embryonic kidneys under the capsule of adult kidney or testis from 3 strains of mice, with the growths being recovered after a defined period. In contradiction of the published data, two distinct morphologies were observed, neither of which resembled classic WT. Both types of morphology were examined with *WT1* and antibodies to developmental markers and it was found that the degree of differentiation was considerably greater in the transplanted tissue than would be expected for WT.

The results as a whole substantiate the importance of *WT1* in tissue undergoing a mesenchyme-to-epithelial transition, particularly during nephrogenesis. The functional significance of *WT1* transcription in tissues outwith this group is less clear. Skeletal muscle is sometimes observed as a component of WT and it is intriguing that the gene is expressed transiently during a stage of muscle development. The data from the mouse points to a key role for *WT1* in the initiation of the cascade of differentiation necessary for normal kidney development. The possible reasons for the failure of the mouse model to produce the Wilms' phenotype are discussed.

## ABBREVIATIONS

| | |
|---|---|
| a | Adenine |
| AMH | Anti-Mullerian hormone |
| AP | Alkaline phosphatase |
| bp | Base pairs |
| BWS | Beckwith-Wiedemann syndrome |
| c | Cytosine |
| C | Cysteine |
| CAM | Chorioallantoic membrane |
| DEPC | Diethyl pyrocarbonate |
| DTT | Dithiothreitol |
| DNA | Deoxyribonucleic acid |
| dpm | Disintergrations per minute |
| EDTA | Ethylenediaminetetra-acetic acid |
| ES | Embryonic stem |
| g | Guanine |
| GBM | Glomerular basement membrane |
| H & E | Haematoxylin and eosin |
| H | Histidine |
| IGF-II | Insulin-like growth factor II |
| ILNR | Intralobar nephrogenic rest |
| kb | Kilobase pairs |
| LIF | Leukaemia inhibitory factor |
| mRNA | Messenger ribonucleic acid |
| N-CAM | Neural cell adhesion molecules |
| NGF | Neural-growth-factor |
| NR | Nephrogenic rest |

| | |
|---|---|
| PBS | Phosphate buffered saline |
| p.c. | Post coitum |
| PCR | Polymerase chain reaction |
| PFA | Paraformaldehyde |
| PFGE | Pulsed-field gel electrophoresis |
| PKD | Polycystic kidney disease |
| PLNR | Perilobar nephrogenic rest |
| RB | Retinoblastoma |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| SSC | Sodium saline citrate |
| t | Thymine |
| TEM | Transmission electron microscope |
| TESPA | 3-aminopropyltriethoxysilane |
| Tris | Tris (hydroxymethyl) aminomethane |
| UV | Ultraviolet |
| WAGR | Wilms' tumour, aniridia, genitourinary abnormalities and mental retardation |
| WT | Wilms' tumour |

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# CHAPTER 1

# INTRODUCTION

Renal malignancies in young children have been recorded since the early nineteenth century, but, as is often the case, Wilms' tumour (WT) derives its name from the author of a much later review of the disease (Wilms, 1899). Many of the early reports suggested an embryonic origin for the tumour, which is also called nephroblastoma, and it was soon recognised that a developmental anomaly lay behind its formation (Nicholson, 1931; Willis, 1958). WT is now considered to arise from the stem cells of the kidney and often includes structures that represent the various stages of nephrogenesis. It is a model for investigating the proposed relationship between malignancy and abnormal differentiation and as such has recently been the subject of intensive research (reviewed by van Heyningen & Hastie, 1992).

The work presented in this thesis examines the expression and possible role in mouse embryogenesis of the gene, *WT1,* that was isolated by positional cloning as a candidate WT predisposition gene, has been implicated in urogenital development and mutations in which have been associated with the tumour (for details see section 1.4). The need for such a study is two-fold: human embryonic material is both more difficult to obtain and less amenable to experimental manipulation than that of the mouse. Here, WT and the isolation and characterisation of *WT1* is discussed, but to set the context in which the tumour forms and the gene is functional, we must first consider the development of the normal mammalian kidney at both the cellular and molecular level and follow this by a brief outline of the formation of the genital system.

## 1.1 Normal development of the kidney

The nephron, which is the basic element of the excretory system of all vertebrates, has been found in the fossil record of the ostracoderms, the oldest known vertebrates (Torrey, 1965). Based on variations in the detailed structure of the nephrons and in their spatial assembly in living animals, the developing renal system can be divided into three distinct organs, the pronephros, the mesonephros and the metanephros. All three develop

from the intermediate mesenchyme that forms two identical blocks of mesodermal tissue running along the dorsal wall of the embryo. These are called the nephrogenic cords in the cervical and thoracic regions and the urogenital ridges in the coelomic cavity, where they widen.

The paired pronephric ducts, later called the mesonephric or Wolffian ducts, extend caudally as development proceeds and are central to the formation of the excretory system. In addition to their role as a drainage channel, these ducts play an important part in the differentiation of both the mesonephros and the metanephros and this will be discussed later. Their origin and subsequent mode of elongation are two features of their formation that have been subjected to investigation (reviewed by Saxen, 1987). The ducts are derived from mesoderm that is distinct from the pronephric mesenchyme, although the most caudal sections may be composed of pronephric tubules that have fused at their distal end. The mechanism of extension differs between vertebrate orders, with cell rearrangement, *in situ* segregation and cell proliferation all being implicated (Poole & Steinberg, 1984). The factors guiding the ducts have also been studied and, in the axolotl embryo at least, the surrounding mesoderm provides a gradient of adhesion (Poole & Steinberg, 1982; Zackson & Steinberg, 1987).

Before going on to describe the formation of the metanephros, it will be helpful to examine briefly the first two renal systems, and Figure 1.1 demonstrates the spatial relationship between the three organs.

### *1.1.1 The pronephros and mesonephros*

Studies of the pronephros have concentrated on the more primitive vertebrates, such as the bony fish and the amphibians, both of which have pronephroi that function in the embryo (reviewed by Saxen, 1987). Here, the pronephric tubules open directly into the coelomic cavity with 'external glomeruli' developing from the coelomic epithelium. In amniotes however, the rostral end of the nephrogenic cord remains rudimentary. Conclusive demonstration of a pronephros in the mouse has not been possible, but in man, the organ is

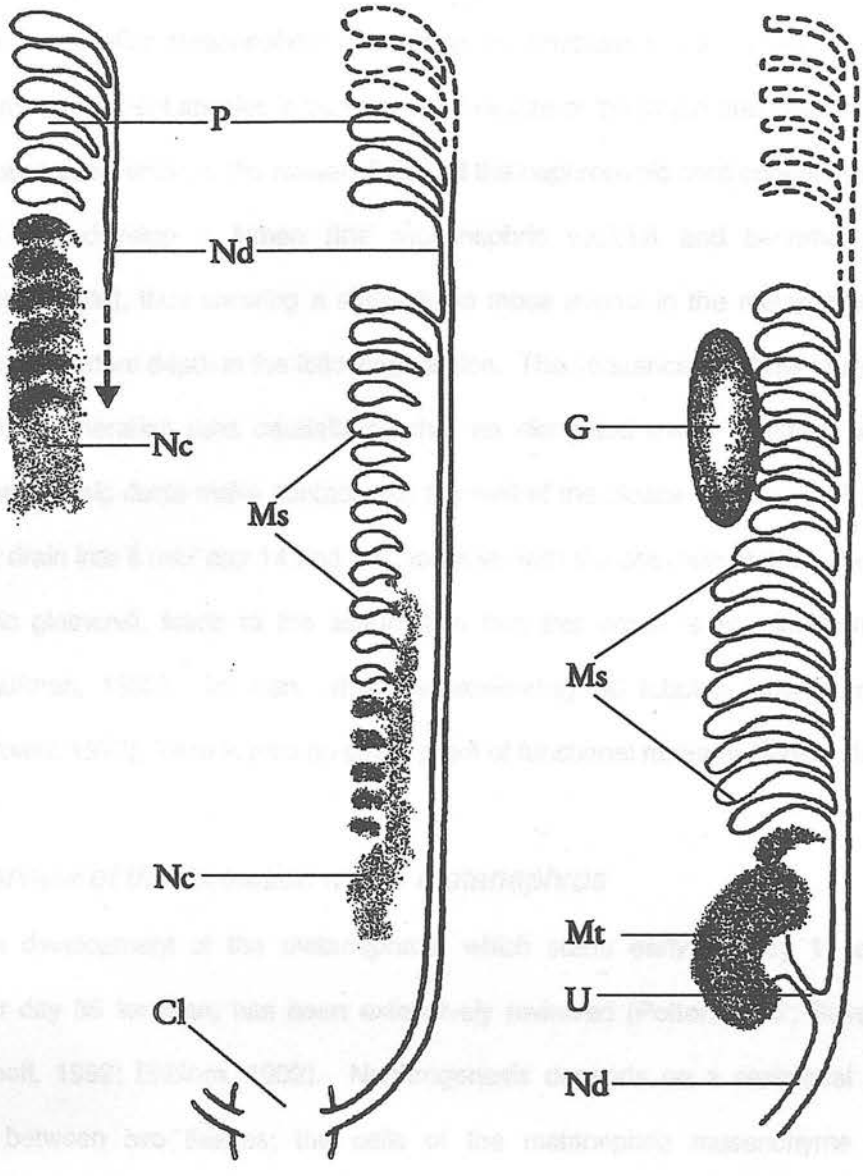



**Figure 1.1**

**An overall scheme of the development of the vertebrate kidney. P, pronephros; Nd, nephric duct; Nc, nephrogenic cord; G, gonad; Ms, mesonephros; Mt, metanephros; U, ureteric bud; Cl, cloaca (Saxen, 1987).**

represented by several transient cell clusters within the intermediate mesoderm of the cranial region that are seen at approximately 22 days of gestation (Potter, 1972).

The mammalian mesonephros is a temporary structure, but a degree of variation is observed among different species in both the relative size of the organ and in its maturity. In the late 9 day mouse embryo, the mesenchyme of the nephrogenic cord condenses, forming aggregates that develop a lumen (the mesonephric vesicle) and become S-shaped (mesonephric tubule), thus showing a similarity to those events in the metanephros which are considered in more depth in the following section. The sequence of tubule formation and subsequent degeneration runs caudally so that an elongated ovoid structure is formed. Although the nephric ducts make contact with the wall of the cloaca by day 10 of gestation, they do not drain into it until day 14 and this, together with the absence of well-differentiated mesonephric glomeruli, leads to the assumption that this organ is non-functional in the mouse (Kaufman, 1992). In man, where approximately 40 tubules form from day 25 onwards (Potter, 1972), there is also no direct proof of functional maturity (Saxen, 1987).

### *1.1.2 Overview of the formation of the metanephros*

The development of the metanephros, which starts early on day 11 of mouse gestation or day 35 for man, has been extensively reviewed (Potter, 1972; Saxen, 1987; Bard & Woolf, 1992; Ekblom, 1992). Nephrogenesis depends on a reciprocal inductive interaction between two tissues; the cells of the metanephric mesenchyme and the epithelium of the ureteric bud (Figure 1.2), which originated from the mesonephric duct. As a result of this interaction the bud elongates into a dense cap of mesenchymal cells and a complex cascade of events is initiated. If the kidney developed in a similar manner to other ducted organs such as the submandibular gland, the branches of the growing ureteric bud would give rise to the entire epithelial component. In the case of the metanephros, however, the parts of the nephron involved in filtration, secretion and reabsorption differentiate from the mesenchyme and it is only the collecting ducts, involved in excretion, that are derived from the ureteric bud.

Figure 1.2 Schematic presentation of the development of the nephron from induction to the formation of the glomerulus within the renal corpuscle (adapted from Ekblom, 1992).

(a) The ureteric bud (U) grows into the metanephric mesenchyme (Mm) and a reciprocal inductive interaction takes place (arrows).
(b) Induction leads to branching of the ureteric bud and further condensation of the mesenchyme, that forms a cap (C) around the tips of the bud. Note that there is evidence at this stage of vascularisation (arrow).
(c) The cells of the condensate become organised into first a renal vesicle (not shown) and then a comma-shaped body. The blood vessel grows into the slit which has formed at the pole furthest from the ureteric bud (arrow).
(d) The S-shaped body is formed by elongation of the comma-shaped body and by the formation of a second slit (arrow). The presumptive visceral (V) and parietal (P) epithelial layers of Bowman's capsule can be distinguished at this stage.
(e) The S-shaped body elongates while the endothelial cells form a capillary bed or glomerlus (G) within Bowman's capsule.
(f) The mature nephron.

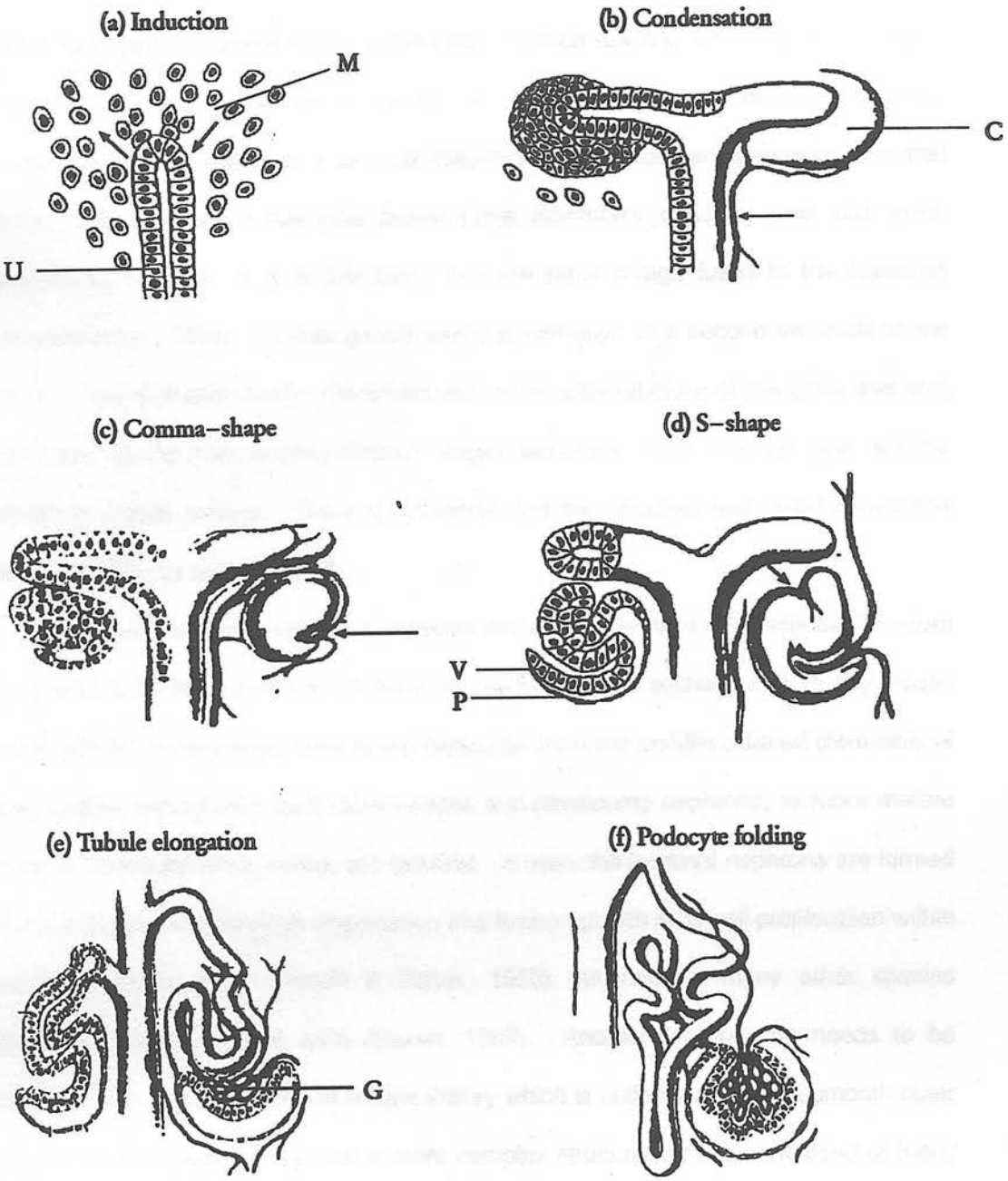

(a) Induction
M
U
(b) Condensation
C
(c) Comma–shape
(d) S–shape
V
P
(e) Tubule elongation
G
(f) Podocyte folding

Figure 1.2

As the bud grows it bifurcates, with the mesenchyme adjacent to it condensing further and segregating into clumps (Figure 1.2). These in turn become organised into pretubular aggregates, or renal vesicles, the cells of which develop a polarity and elongate but continue to have a uniform ultrastructure. A comma-shaped body develops from the renal vesicle by the formation of a slit that divides the future tubular epithelium from that associated with the glomerulus (the presumptive Bowman's capsule) and into which endothelial cells migrate. It is at this stage that the renal anlage fuses to the collecting system (Jokelainen, 1963). Further growth and the formation of a second slit leads to the production of the S-shaped body. Diversification in the ultrastructure of the cells, that was first apparent during the comma-shaped stage, becomes more marked and will be considered in a later section. The loop of Henle and the proximal and distal convoluted tubules are created by further growth.

Within the developing kidney, a temporal and spatial gradient of differentiation exists so that all stages of nephrogenesis can be seen in a longitudinal section. In a 15 day mouse embryonic kidney, for example, there is a progression from the undifferentiated stem cells of the outer cortex, through the early condensates and developing nephrons, to more mature nephrons at the border of the cortex and medulla. In man, the terminal nephrons are formed between the 32nd and 36th week of gestation and further growth is by cell proliferation within the existing tubules (Osathanondh & Potter, 1963), whereas in many other species nephrogenesis continues after birth (Saxen, 1987). Another feature that needs to be considered is that, in contrast to the mouse kidney which is unilobar and has a smooth outer surface, the human metanephros has a more complex structure as it is composed of many lobes. The multilobed structure is particularly striking in human material that has been sectioned where the stem cells of the outer cortex can in fact lie deep within the organ.

### *1.1.3* In vitro *development of the kidney*

The need for a technique that allowed the controlled manipulation of the metanephros as it formed led to the development of an *in vitro* organ culture system for the

mouse kidney (Grobstein, 1953a,b; 1956) and this has subsequently been modified and improved (Saxen, 1987). Much of the work that will be described in the following sections resulted from experiments carried out using either whole-organ or transfilter culture and these will be considered in turn.

For whole-organ culture, metanephric rudiments from mouse embryos of 11.5 days of gestation are dissected out and placed on porous filters that are maintained at the air-liquid interface of a dish containing an appropriate medium. The surface tension ensures that the rudiments remain covered by a thin layer of medium and if good differentiation is required, these are then cultured for about 6 days. Development will proceed to an advanced stage with branching of the collecting system and the mesenchyme-to-epithelium transition occurring to produce mature tubular structures (Bard & Ross, 1991).

In the transfilter system, the metanephric rudiments from 11 day embryos (where induction has not yet occurred) are isolated and the mesenchyme and ureteric bud components separated either enzymatically or mechanically. The ureteric bud, which does not work efficiently across a filter, is replaced by a heterologous inducer such as the embryonic spinal cord. The tissues are placed opposite each other, on the upper and lower surface of a porous filter, immobilised with agar, and cultured in conditions similar to the whole-organ for up to 5 days. The degree of morphological differentiation seen in this system has been compared to that *in vivo* and found to be remarkably similar, including the development of proximal tubules with a well-defined brush border, distal tubules and renal corpuscle-like bodies with podocytes, but no endothelial cells (Ekblom *et al.*, 1980a; 1981a; Bernstein *et al.*, 1981). This system is particularly useful for experiments involving cell or tissue interaction and has provided unequivocal proof that the epithelial cells of the nephron are derived from the mesenchyme.

### *1.1.4 Induction of the metanephric mesenchyme*

The study of the mechanisms underlying the differentiation of the mesenchyme into epithelium has been facilitated by the ability of the kidney to develop *in vitro*. The transfilter

technique has been used to investigate the initial interaction and has demonstrated that, if the mesenchyme is isolated from the ureteric bud and cultured alone, it fails to grow very well or to differentiate (Grobstein, 1955). A variety of tissues in addition to the natural source have been found to induce (or trigger) the metanephric mesenchyme into forming tubules (Grobstein, 1955; Unsworth & Grobstein, 1970; Saxen, 1987). In contrast, no other mesenchyme has been shown to produce nephrons (Saxen, 1970) and it has been concluded that the metanephric mesenchyme has a pre-determined kidney bias, with the induction being permissive rather than directive (Saxen, 1977). These two categories have been defined as follows: for directive induction, the embryonic cell has more than one developmental option with the choice being affected by extracellular factors; in permissive induction, however, the cell is already committed to a particular developmental pathway, but requires a stimulus to express this new phenotype (Saxen, 1977). The inductive stimulus need not be long and exposure of the mesenchyme to the inducer for 24 h has been shown to be sufficient to lead to the formation of the epithelial cells of Bowman's capsule and the proximal tubule (Saxen & Lehtonen, 1978). Using electron microscopy, it has been demonstrated that, during transfilter experiments, cytoplasmic processes from the cultured tissues penetrate the pores of the filter and become closely apposed. This has led to the suggestion that cellular interaction, rather than long range diffusion of a signal substance, is responsible for induction (Wartiovaara *et al.*, 1974).

The transfilter experiments established that embryonic spinal cord was a more efficient inducer than the ureteric bud and this led to the idea that nerves may be involved in the process. Nerve cells have been found in undifferentiated kidneys running along the ureter, with processes going out into the mesenchyme (Sariola *et al.*, 1988a) and antibodies that prevent their binding to mesenchymal cells disrupt differentiation (Sariola *et al.*, 1989). The most powerful evidence to support this hypothesis, however, is the demonstration that antisense oligonucleotides that bind to the neural-growth-factor (NGF) receptor mRNA suppress receptor synthesis and also prevent nephrogenesis (Sariola *et al.*, 1991).

The changes that occur as the induced mesenchyme undergoes epithelialisation have been studied extensively using immunohistochemistry. Before induction, the cells are not in direct contact and are surrounded by a matrix that includes fibronectin (Ekblom, 1981) and the interstitial collagens I and III (Ekblom *et al.,* 1981b). In addition, the neural cell adhesion molecules (N-CAM), a family of related glycoproteins, are expressed on the cell surface (Klein *et al.,* 1988). As the induced mesenchyme forms aggregates, fibronectin and collagens I and III disappear rapidly, so allowing closer cell contact (Ekblom, 1981; Ekblom *et al.,* 1981b), whereas N-CAM remains on the epithelial cells until polarisation, as assayed by the formation of a lumen, is evident (Klein *et al.,* 1988). The cell surface proteoglycan, syndecan, which is a receptor for interstitial matrix material and thus acts as an adhesion molecule between cells, has been detected first in the mesenchyme surrounding the unbranched ureteric bud, then, as this bifurcates, in the entire metanephric mesenchyme; it therefore seems to be an early response to induction (Vainio *et al.,* 1989). Proteins specific for desmosomes, which are intercellular junctions associated with epithelial sheets, have been found in the initial aggregate and may well play a role in strengthening the contact between cells of the early kidney tubule (Garrod & Fleming, 1990).

The formation of a basement membrane, or basal lamina, is a characteristic feature of epithelial cells and electron microscopy studies have shown that in the kidney it starts to appear around the renal vesicle shortly after this structure aggregates and is complete by the time the S-shaped body forms, even extending into the slits (Jokelainen, 1963). The main constituents of the basal lamina are laminin, heparin sulphate proteoglycan and collagen IV and these have been detected in the mesenchymal aggregates as a punctate pattern, becoming confined to the outer edge of the renal vesicle and giving rise to a sharp continuous band around the more mature tubules (Ekblom, 1981; Ekblom *et al.,* 1980b; 1981b; Bonadio *et al.,* 1984). 12 h after the completion of induction, the cell adhesion molecule, uvomorulin, is produced (Vestweber *et al.,* 1985) and is co-expressed with N-CAM for a short period (Klein *et al.,* 1988). In the cytoskeleton, the intermediate filament vimentin,

uniformly present in the uninduced mesenchyme, is replaced in the forming tubules by cytokeratin, a marker for epithelial cells (Lehtonen *et al.*, 1985).

Tenascin, a mesenchymal matrix protein (Chiquet-Ehrismann *et al.*, 1986) has been observed around the condensates and S-shaped bodies, so supporting the hypothesis that this molecule is involved in epithelial-mesenchymal interactions (Aufderheide *et al.*, 1987). A similar role has been indicated for the cell surface glycolipid, $G_{D3}$, because monoclonal antibodies that react with $G_{D3}$ prevent branching of the collecting system and the mesenchyme-to-epithelium transition (Sariola *et al.*, 1988b)

It is an intriguing observation that the aggregates form from mesenchyme that is already condensed. *In vitro* studies have shown that rudiments grown on a substrate to which they adhere weakly form fewer nephrons than those grown on more sticky substances and these results suggest that there is a mechanical aspect to this condensation rather than a prepattern (Bard, 1990a).

We now have some insight into the neural basis of induction and the subsequent changes in the molecular phenotype of the differentiating mesenchyme are well documented, but two important questions remain to be fully addressed. The first of these is whether all of the metanephric mesenchyme is induced at once, while the second again concerns the mesenchyme which has two developmental options; either to become interstitial mesenchyme or to be transformed into epithelium, and it is not clear if these are both derived from induced tissue. These problems are fundamental to the understanding of the formation of WT and, as we shall see later, have yet to be answered satisfactorily.

### *1.1.5 Formation of the collecting system*

The interaction between the mesenchyme and the duct is reciprocal, with the bud failing to branch in organ culture if the mesenchyme is removed (Grobstein, 1955), and seems to be specific as no heterologous mesenchyme has been reported to support branching (Saxen, 1970). Little is known about the mechanisms underlying branching in the

kidney but an analogous organ, the submandibular salivary gland, has been studied extensively (reviewed by Bernfield *et al.*, 1984; Bard, 1990b). In this system, it has been proposed that the mesenchyme degrades and then remodels the epithelial basal lamina and the branching pattern seems to result from the interaction between the epithelium and the mesenchyme. Until we have a better understanding of the process in the kidney, the salivary gland is the best model, but the differences in the formation of the two organs limit the conclusions that can be drawn.

The branching pattern of the ureteric bud in the human embryo has, however, been described in detail and the process divided into 4 periods (Osathanondh & Potter, 1963) with a similar course of events occurring in the rat (Neiss, 1982). The first of these periods is marked by the dichotomous branching of the ureteric bud at the widened tips or ampullae. There are two types of dichotomous branching; symmetric and asymmetric, with the latter occurring when the two branches differ in their subsequent development. In the second stage, the collecting system has stopped branching but the ampullae have retained their ability to induce renal vesicle formation. This leads to more than one nephron fusing to each tip, with the more mature ones shifting position and thus forming an arcade (Figure 1.3). During the third period the collecting duct grows past the attachment point of the arcade and although it does not branch again it does induce a terminal set of nephrons (Figure 1.3). The last period is characterised by the termination of induction and the cessation of nephron formation due to the disappearance of the ampullae at the end of the previous stage.

Studies of human development have also shown that the early branches of the ureteric bud dilate soon after the formation of the first glomeruli to form the renal pelvis, the calyses and the papillae (Potter, 1972). A consequence of this is that the collecting ducts that drain into the papillary ducts are in fact up to 11 generations removed from the initial bifurcation of the ureteric bud (Potter, 1972).

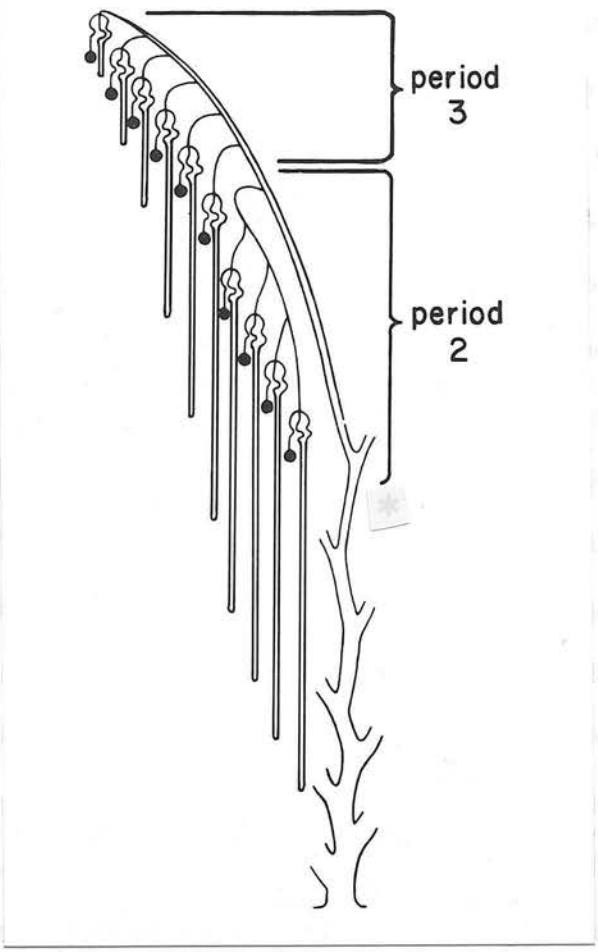



Figure 1.3. The arrangement of the human nephrons at birth, illustrating periods 2 and 3 of the development of the collecting system. Four deeper nephrons constitute an arcade, formed during period 2, while the six cortical nephrons are directly connected to the collecting duct, a characteristic feature of period 3 (Osathanondh & Potter, 1963).

### *1.1.6 The renal corpuscle and angiogenesis*

The normal development of the kidney depends on a fourth cellular component, the endothelial cells of the vascular system. These cells form a capillary bed, or glomerulus, within Bowman's capsule and this is collectively termed the renal corpuscle. The origin of the endothelial cells has been the cause of much controversy and it was Potter (1965) who first put forward the suggestion, now widely held, that these cells migrate into the kidney from an external source. Indirect evidence for this hypothesis was provided by the demonstration of avascular renal corpuscles in the transfilter system (Bernstein *et al.,* 1981; Ekblom, 1981), but direct proof awaited experiments using the avian chorioallantoic membrane (CAM). Mouse metanephric rudiments were grown on the quail CAM and, based on the distinctive nuclear structure of the quail cells, it was concluded that the capillaries were derived from the host tissue and thus had an external origin (Ekblom *et al.,* 1982). A further study, in which separated uninduced and experimentally induced metanephric rudiments were grafted onto the quail CAM showed that the stimulation of endothelial cell ingrowth required the interaction between the ureteric bud and the mesenchyme (Sariola *et al.,* 1983). The guiding signal for the migration of the capillaries is unknown, but the embryonic kidney has been shown to produce an angiogenic factor (Risau & Ekblom, 1986).

The section of the nephrogenic epithelium that will give rise to Bowman's capsule can, as previously mentioned, be identified at the stage of the comma-shaped body. Shortly afterwards (during the S-shaped phase) a further diversification occurs so that two cell types can be distinguished; the future visceral epithelium (or podocytes) and the parietal epithelium (see Figure 1.2). Morphologically, the cells of the visceral layer are more columnar than those of the parietal layer and in addition have a different lectin-binding capacity (Ekblom *et al.,* 1981a) and express ZO-1, a protein first detected in tight junctions, at a higher level than other cells of the nephron (Schnabel *et al.,* 1990). In the mature renal corpuscle, the podocytes are closely apposed to the endothelial cells and have a stellate appearance, with radiating primary processes that give rise to secondary ones called pedicels or foot

processes. These interdigitate with those of other podocytes to create a system of intercellular clefts called slit pores that are involved in filtration.

The podocytes are separated from the endothelial cells by a basal lamina, the glomerular basement membrane (GBM), which is the main filtration barrier of the kidney. Experiments using CAM grafts have demonstrated that the GBM is derived from both cell types and thus has a dual cellular origin (Sariola *et al.*, 1984). The source of another component of the renal corpuscle, the mesangial cells, has also been investigated. These cells, whose function is not clear, were not observed in the avascular renal corpuscles formed *in vitro* (Bernstein *et al.*, 1981) and CAM graft experiments have shown that these cells are derived from the host tissue suggesting they are either vascular derivatives or migrate into the kidney rudiment with the endothelial cells (Sariola *et al.*, 1984).

## 1.2 Development of the genital system

From a functional point of view, the urinary and genital systems are very different, but they do have a close developmental and anatomical connection, being formed from a common mesodermal ridge, the intermediate mesoderm. It is therefore of no surprise that WT is often associated with genital abnormalities and a brief description of normal development follows (reviewed by Moore, 1982; Kaufman, 1992).

Although the sex of the mammalian embryo is predestined chromosomally at the time of fertilisation, the morphological distinction is only possible from day 12.5 of gestation in the mouse or from 7 weeks in the case of the human embryo. Prior to this, the gonads, which will go on to differentiate into either the ovaries or the testes, are said to be 'indifferent'. They first appear on day 10 (5th week in the human) as a thickening of the coelomic epithelium with an underlying condensation of the mesenchyme on the lateral side of each mesonephros and are termed the genital or gonadal ridges. The proliferating

coelomic epithelium goes on to invade the subjacent mesenchyme, forming the irregularly shaped primary sex cords.

In male embryos, the primary sex cords condense to form the seminiferous cords and these give the developing testis its characteristic striped appearance. These cords, which subsequently lose contact with the surface epithelium, develop a lumen, while the mesenchyme between them gives rise to the interstitial cells of Leydig. The seminiferous tubules are made up of two cell types: the Sertoli cells derived from the surface epithelium and which predominate in the embryonic organ and the spermatogonia which differentiate from the primordial germ cells. As it enlarges, the testis becomes separated from the regressing mesonephros and becomes suspended from its own mesentery, the mesorchium. In the female embryo, the primary sex cords degenerate, but the surface epithelium continues to proliferate, giving rise to the secondary or cortical cords. These remain close to the surface and incorporate the primordial germ cells. The cortical cords break down in turn to form isolated cell clusters or follicular cells which surround the germ cells (oogonia).

During the initial 'indifferent' period, both sexes have two pairs of genital ducts: the mesonephric (Wolffian) and paramesonephric (Mullerian) ducts. The formation of the mesonephric duct has already been described (see section 1.1), with the paramesonephric duct being formed from an invagination of the coelomic epithelium on the lateral surface of the urogenital ridge, possibly induced by the presence of the subjacent mesonephric duct. In the male embryo, the testes produce anti-Mullerian hormone (AMH) that causes the paramesonephric ducts to regress and stimulates the mesonephric ducts to form the epididymis, ductus deferens and the ejaculatory ducts of the adult. In the female embryo, the mesonephric ducts regress in the absence of the stimulus provided by the testes and the rostral end of the paramesonephric duct opens into the coelomic cavity with a funnel-like structure, which will become the oviduct in the adult. As the duct runs caudally in the human embryo, it comes into close contact with the corresponding duct of the opposite side and these later fuse to give rise to the uterus and upper vagina. In most other mammalian

species, including rodents, however, the two paramesonephric ducts remain as separate entities along most of their course.

## 1.3 Wilms' tumour

Having set out the developmental context in which WT stands, we will now consider the clinical background of the malignancy (reviewed by Greenwood & Holland, 1984; Pritchard-Jones & Hastie, 1990) before going on to discuss the genetic aspects. WT is one of the most common solid tumours occurring in infants, affecting on average 1 in 10,000 live births and seems to have a relatively uniform distribution throughout the world, although there is evidence of variation between certain ethnic groups. The tumour is very rare in the new-born, with the median age at diagnosis being between 3 and 4 years and 90% of cases presenting before the age of 7. Most cases of WT are sporadic and unilateral, but between 5% and 10% are bilateral and a further 1% show familial transmission, where it is inherited as an autosomal dominant trait with variable penetrance (Matsunaga, 1981). When the age of onset for these groups was separated, the unilateral cases were found to be significantly older than those presenting with bilateral tumours (Breslow *et al.*, 1988).

Although the tumour has often grown to a large size before diagnosis, it is generally still confined to the kidney with local extension being more frequent than distant metastases. Where such distant metastic spread of the disease does occur, it is usually to the lungs by way of the bloodstream. Advances in medical treatment mean that the majority of cases can now be cured, but successful treatment is dependant to a degree on the histological profile of the tumour.

### *1.3.1 Histopathology of Wilms' tumour*

The classic triphasic morphology, which has been described for many of the tumours, involves three cell types that are all derivatives of the kidney stem cell, namely the

blastemal, stromal and epithelial cells. There is, however, a wide variation in the histological appearance and tumours may be composed predominantly of only one or two cell types. Details of the classification of the tumour will not be considered here (for description see Kidd, 1984), but it should be pointed out that two subtypes have been designated as being distinct from WT; clear-cell sarcoma and malignant rhabdoid tumour, both of which have a high mortality rate (Beckwith, 1983). In addition, the association of anaplasia (cells with large hyperchromatic nuclei and abnormal mitotic figures) with WT has been recognised as a marker of an unfavourable prognosis (Beckwith & Palmer, 1978).

The origin of the three cellular components found in triphasic tumours has been examined in the context of normal nephrogenesis and based on a review of ultrastructural and immunohistochemical studies (Mierau *et al.,* 1987). The blastemal cells have been proposed to represent the condensed mesenchyme of the kidney during the formation of the initial renal aggregates. The histogenesis of the stromal component has not yet been resolved, although there are indications that it corresponds to mesenchyme that has yet to undergo aggregation, rather than to the connective mesenchyme of the mature kidney. Alternatively, it may be the product of the malignant blastema (Brown *et al.,* 1989). The primitive epithelial tubules, that are often observed, are thought to represent an early stage in nephrogenesis, such as the normally transient renal vesicle, and only in very rare cases are structures indicative of late nephrogenesis (i.e. the proximal tubule) seen. In some examples, 'glomeruloid-bodies' form and these seem to be attempts to differentiate into the renal corpuscle. There is also evidence that a proportion of the epithelial tubules are derivatives of the collecting duct system and these may originate from the adjacent normal kidney. In addition, components such as striated muscle, cartilage, bone, adipose tissue and neural tissue are sometimes seen and these are usually associated with areas of stromal cells. These heterologous elements are not normal derivatives of the kidney stem cell and are considered to be produced as a result of inappropriate differentiation.

Lesions that may be the precursor of WT, have been found in up to 44% of kidneys removed due to unilateral WT and in nearly 100% of those associated with bilateral cases of

the disease (Bove & McAdams, 1976; Beckwith *et al.*, 1990). These lesions, or nephrogenic rests (NR), are foci of embryonal cells that are thought to have retained their pluripotency and have been divided into intralobar (ILNR) and perilobar (PLNR) categories (Beckwith *et al.*, 1990). The former occur throughout the body of the kidney and are mainly stromal, with small areas of blastemal cells and the occasional epithelial component, suggesting an early developmental aberration. PLNR are well-defined islands of predominately blastemal cells at the periphery of the lobes of the kidney and are thought to represent a disturbance during late nephrogenesis. Multiple or diffuse NR can occur and this condition has been termed nephroblastomatosis. The histological appearance of the tumours associated with the two classes of lesions is also different: ILNR are found in conjunction with predominately stromal tumours and frequently contain heterologous elements, whereas PLNR seem to be linked to tumours with a later onset that often show evidence of epithelial differentiation. PLNR are, however, also found in 1% of all infant post-mortems (Bennington & Beckwith, 1975), which suggests that, if they are a premalignant lesion, the majority do not progress to WT.

### *1.3.2 Congenital abnormalities*

Although most cases of WT are unilateral and sporadic, several congenital abnormalities are known to be associated with the tumour (Miller *et al.*, 1964; Pendergrass, 1976) and these have proved invaluable in understanding the disease. There is a significant increase in the number of malformations involving the urogenital system, with those of the urinary tract including duplex and horse-shoe kidneys, double ureters and renal agenesis (Breslow & Beckwith, 1982). Genital abnormalities seem to be more common in males and range from cryptorchidism (undescended testis) and hypospadias (abnormal penile development) (Breslow & Beckwith, 1982), to the more severe phenotype of gonadal dysgenesis (Rajfer, 1981).

It was the surprisingly high proportion of aniridia patients (absence of the iris), who subsequently developed WT (Miller *et al.*, 1964), that first suggested a locus for a gene. Approximately one third of sporadic aniridia cases later develop WT (Fraumeni & Glass,

1968) and 1 in 100 of WT cases are born with aniridia (again mainly sporadic)(Breslow & Beckwith, 1982) as compared to an incidence in the general population of 1:64,000 (Shaw *et al.,* 1960). Many of these patients have genitourinary abnormalities and are mentally retarded and this collective phenotype is known as the WAGR syndrome (Wilms, Aniridia, Genitourinary abnormalities and mental Retardation). Constitutional deletions spanning part of the short arm of one homologue of chromosome 11 (the distal half of band 11p13) were cytogenetically observed in a number of patients with the WAGR syndrome (Riccardi *et al.,* 1978; Franke *et al.,* 1979). The WT gene from this locus has recently been isolated by positional cloning (Call *et al.,* 1990; Gessler *et al.,* 1990) and will be discussed in detail in a later section.

Three further syndromes are associated with WT and these are; Beckwith-Wiedemann (BWS), Denys-Drash and Perlman's syndrome. BWS is characterised by fetal gigantism, that is often asymmetric giving rise to hemihypertrophy, and also by abnormal closure of the abdominal wall (Wiedemann, 1964; Beckwith, 1969). A high proportion (7%) of patients with BWS develop abdominal tumours, the most common of which is WT comprising more than half of such tumours (Wiedemann, 1983). The Denys-Drash syndrome encompasses pseudohermaphroditism, intrinsic renal parenchymal disease (nephrotic syndrome) and WT (Denys *et al.,* 1967; Drash *et al.,* 1970) and is characterised by a specific glomerular lesion, with shrunken glomerular tufts surrounded by hypertrophied epithelial cells (Habib *et al.,* 1985). Perlman's syndrome is the phenotype comprised of nephroblastomatosis, fetal gigantism and cryptorchidism in males (Perlman *et al.,* 1973). There is a high incidence of stillbirth and neonatal death in this syndrome, but those that survive subsequently develop WT.

### *1.3.3 Genetics of Wilms' tumour*

Malignancies of early childhood have become the focus of research into the genetic changes associated with cancer because they are thought to need fewer mutational steps than those with a later onset (reviewed by Haber & Housman, 1991). This idea is based on

the 'two-hit' hypothesis, a model that had originally been proposed for retinoblastoma (RB), a childhood eye tumour (Knudson, 1971), but was subsequently modified for WT (Knudson & Strong, 1972). Statistical analysis was carried out on a large collection of tumours with the age of onset and the number of malignant growths per individual being compared, so that the number of rate limiting events could be estimated. The 'two-hit' hypothesis predicts that in hereditary cases, the first mutation is present at the prezygotic (germinal) stage and therefore exists in all cells. Only one additional mutation is then needed in a cell of the target organ to give rise to a tumour. Non-hereditary tumours, on the other hand, are postulated to be caused by two postzygotic (somatic) mutations occurring in the same cell. As the probability is very small that two somatic mutations would occur in more than one cell, this model predicts that multiple and bilateral tumours are hereditary cases. The earlier onset of bilateral tumours also reflects the idea that the first mutation is carried in the germline.

Although Knudson's model only went so far as to estimate the number of rate limiting steps, it has since become synonymous with the idea that mutations disrupting both alleles of a single gene are necessary and sufficient for the formation of the tumour. The suggestion that nephroblastoma is due to a loss of gene function has led to it being classed with the so-called 'anti-oncogenes' or 'tumour suppressor' genes (Klein, 1987: Weissman *et al.*, 1987; Marshall, 1991). This term is, however, misleading because it implies that the gene is only involved in preventing uncontrolled cell proliferation and fails to acknowledge any role for it in normal development.

RB has been shown to conform to the 'two-hit' hypothesis with the isolation of *RB1*, the gene at 13q14, that is inactivated in cases of this tumour (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). For WT, karyotype analysis of WAGR patients had demonstrated loss of genetic material corresponding to the first-hit predicted by Knudson. Polymorphic DNA probes were then used to show that in sporadic tumours there was loss of heterozygosity for the short arm of chromosome 11 (Fearon *et al.*, 1984; Koufos *et al.*, 1984; Orkin *et al.*, 1984; Reeve *et al.*, 1984), that was restricted to 11p13 in a couple of cases

(Wadey *et al.*, 1990). In the case of WT, however, the 'two-hit' hypothesis is not in itself sufficient to explain the complex nature of the malignancy because there seems to be at least two more genes, in addition to the one at 11p13, implicated in the development of the tumour.

A locus on the short arm of chromosome 11 that is closer to the telomere was indicated by loss of heterozygosity in the majority of cases of the tumour examined for markers within 11p15 that did not involve 11p13 (Mannens *et al.*, 1988; Reeve *et al.*, 1989). Additional evidence stems from the fact that in several individuals with sporadic BWS a constitutional duplication of 11p15 has been reported (Waziri *et al.*, 1983; Turleau *et al.*, 1984a) and rare cases of familial BWS have been shown to be tightly linked to the 11p15 locus (Koufos *et al.*, 1989; Ping *et al.*, 1989). The demonstration of trisomy at 11p15, with most cases involving duplication of the paternal allele, indicates that a different mechanism from chromosome deletion may be involved. Furthermore, several cases have now been described where instead of duplication of 11p15 there was no maternally inherited copy, with both alleles being contributed by the father (Henry *et al.*, 1991), suggesting that differential imprinting of the paternal allele together with gene dosage may be the mechanism involved in BWS. It is of interest in this context that the gene for the insulin-like growth factor II (*IGF-II*) is located at 11p15 (Bell *et al.*, 1985) and that this gene is known to be imprinted in the mouse, with only the paternal allele being expressed during normal development (DeChiara *et al.*, 1990; 1991). To add to the genetic complexity, familial cases of WT have been shown by linkage analysis to map outside chromosome 11 (Grundy *et al.*, 1988; Huff *et al.*, 1988; Schwartz *et al.*, 1991), indicating that a third gene locus may be involved.

The question arises as to whether tumours resulting from genetic loss at 11p13 differ from those involving either 11p15 or the familial locus. Although there is little data on this at present, it has been shown that the NR associated with BWS differ from those seen in conjunction with WAGR (Beckwith *et al.*, 1990). ILNR have been found to be strongly associated with aniridia and Denys-Drash syndrome, while PLNR have been linked with hemihypertrophy and BWS.

## 1.4 Isolation of *WT1*, the gene at 11p13

The region containing the 11p13 WT predisposition gene was defined using somatic cell hybrids containing fragments of human chromosome 11 in a Chinese hamster cell background (Kao *et al.*, 1976). It was found to lie between the erythrocyte catalase gene (*CAT*) and the β subunit of the follicle-stimulating hormone (*FSHB*) (Junien *et al.*, 1980; van Heyningen *et al.*, 1985; Glaser *et al.*, 1986). That the aniridia (*AN2*) and WT genes, although genetically close, are actually separate genes was indicated by a patient with a constitutional deletion of 11p13 who had nephroblastoma and cryptorchidism, but did not have aniridia (Turleau *et al.*, 1984b) and this was subsequently confirmed (Davis *et al.*, 1988a; Gessler *et al.*, 1989). The order of the genes was established to be centromere-CAT-WT-AN2-FSHB-telomere. A large number of DNA markers for this interval were generated (Porteous *et al.*, 1987; Bickmore *et al.*, 1988; Davis *et al.*, 1988b; Gessler *et al.*, 1989) and pulsed-field gel electrophoresis (PFGE) was used to generate long-range physical maps of the region (Compton *et al.*, 1988; Gessler & Bruns, 1989; Davis *et al.*, 1990; Rose *et al.*, 1990). A single sporadic tumour was described that contained two unequal deletions (Lewis *et al.*, 1988) and this narrowed the interval to 350 kb (Compton *et al.*, 1990; Rose *et al.*, 1990). The homologous loss in this tumour was also the first direct evidence that the gene at this locus is a 'tumour suppressor'. The region of genitourinary abnormalities maps to the same 350 kb stretch and could either be one gene with pleiotropic effects or two separate but closely linked genes (van Heyningen *et al.*, 1990).

The candidate WT gene was isolated independently by two groups using different experimental approaches. Call *et al.* (1990) isolated several genomic clones (they used a cosmid library prepared from a somatic cell hybrid containing the short arm of chromosome 11) that mapped to the WAGR region. They went on to identify one clone that showed strong cross-species hybridisation to hamster and mouse genomic DNA and to RNA from baboon kidney and spleen. This genomic probe was then used to screen human cDNA libraries derived from adult kidney, an embryonic kidney cell line and a pre-B cell line.

Several overlapping cDNA clones were identified with the longest one, WT33 (2.3 kb), being derived from the pre-B cell line library.

PFGE analysis of the 11p13 region had previously revealed several CpG islands (Compton *et al.*, 1988; Bickmore *et al.*, 1989; Gessler & Bruns, 1989), which are structures frequently associated with the 5' ends of expressed sequences (Bird, 1986). Starting from one of these CpG islands, the second group (Gessler *et al.*, 1990) isolated 4 neighbouring islands using a rare-cutting restriction-enzyme 'jumping' library. The genomic regions flanking these islands were examined and two showed cross-species conservation. The RNA transcript from one of these genomic clones was found to be expressed specifically in the embryonic kidney and was used to isolate a 3 kb cDNA (LK15) from a human fetal kidney cDNA library. LK15 corresponds to the gene cloned by Call and co-workers and that is now known as *WT1* (Haber *et al.*, 1990).

### *1.4.1 Structure of* WT1

The *WT1* gene contains 10 exons that span 50 kb of DNA and encodes a mRNA transcript of 3 kb (Call *et al.*, 1990; Gessler *et al.*, 1990; Haber *et al.*, 1991). Sequence analysis of the cDNA that was cloned, predicted that the polypeptide product has two functional domains: four carboxy-terminal zinc fingers and a proline-glutamine rich amino-terminal region (Call *et al.*, 1990; Gessler *et al.*, 1990; Figure 1.4). This, together with the nuclear location of the protein (Pelletier *et al.*, 1991a), suggests that WT1 is a transcription factor and thus has a role in the transcriptional control of other genes. In order to understand the possible role of WT1 we must first discuss transcription factors and then go on to look at zinc-finger containing genes.

Transcription factors are sequence-specific polypeptides that have been grouped into families on the basis of their DNA-binding motifs and show a high level of conservation between species. In many cases a second region, the transactivation domain, has been recognised and areas rich in proline and glutamine have been implicated in this role (Mitchell

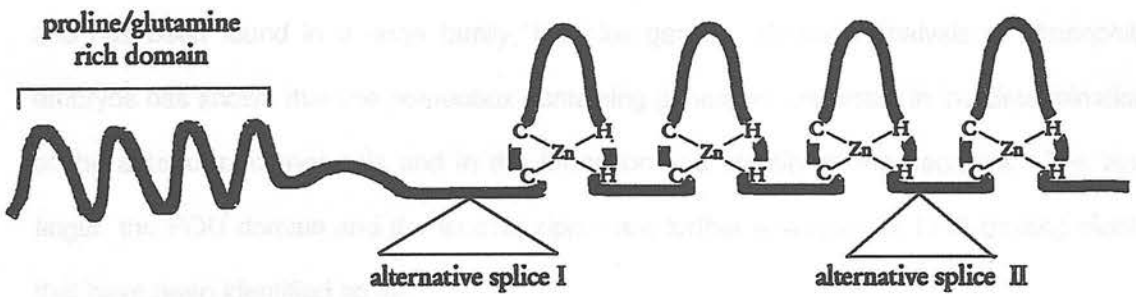



Figure 1.4. Diagrammatic representation of the *WT1* gene product. *WT1* encodes a protein that contains four zinc fingers at the carboxy-terminus and a proline-glutamine rich region at the amino-terminus. Two alternatively spliced segments are found in the *WT1* transcript, resulting in a combination of four distinct mRNA species. Splice I encodes 17 amino acids, while splice II encodes 3 amino acids that disrupt the linker region between zinc fingers 3 and 4 (Haber & Buckler, 1992).

& Tjian, 1989). The first regulatory factors to be identified, the prokaryotic DNA-binding proteins, such as the lambda repressor, have a common helix-turn-helix motif that has been shown to interact with the DNA (Pabo & Sauer, 1984). This general structure is shared by many of the homeotic genes of *Drosophila* and these contain a nucleotide sequence called the homeobox that encodes the homeodomain, a 60 amino acid region with DNA-binding specificity (reviewed by Gehring *et al.,* 1990). The homeobox is conserved in vertebrates and has been found in a large family, the *Hox* genes. Mutation analysis of *Drosophila* embryos has shown that the homeobox containing genes are important in the determination of the anterior-posterior axis and in the formation and identity of the segment. The zinc finger, the POU domain and the leucine zipper are further examples of DNA-binding motifs that have been identified so far.

Zinc-finger proteins can be divided into two classes on the basis of the number and position of the cysteine (C) and histidine (H) residues which co-ordinate zinc chelation (reviewed by Evans & Hollenberg, 1988). The $C_2H_2$ class, to which WT1 belongs, is exemplified by the *Xenopus laevis* transcription factor TFIIIA, the first zinc-finger protein to be described (Miller *et al.,* 1985). This family contains pairs of C and H separated by a loop of twelve amino acids, with at least two tandem repeats linked by between 7 and 8 amino acids. The second class, the Cx proteins, have a variable number of C residues and include the yeast *GAL4* gene (Pan & Coleman, 1990) and the steroid receptor family (Hard *et al.,* 1990). The sequence of *WT1* has been compared to that of other zinc-finger genes and found to be most homologous to a class of mammalian early growth response genes, the *EGR* (human) and *Krox* (mouse) families, that are expressed during the G0 to G1 transition in cultured cells (Chavrier *et al.,* 1988; Joseph *et al.,* 1988; Sukhatme *et al.,* 1988). The interaction between the zinc fingers of *EGR-1* and DNA has been studied in detail using X-ray crystallography (Pavletich & Pabo, 1991) and this has allowed the functional significance of *WT1* mutations to be examined (see section 1.4.2). Other examples of zinc-finger encoding genes include general transcription factors such as *Sp1* (Gidoni *et al.,* 1984) and

the *Drosophila* segmentation genes *Hunchback* (Tautz *et al.*, 1987) and *Kruppel* (Rosenberg *et al.*, 1986).

The genomic structure of *WT1* has also been examined in some detail (Haber *et al.*, 1990; 1991). Each of the zinc fingers is encoded by a distinct exon and is separated from the next finger by a short intron. Another feature of the *WT1* gene is that it produces 4 distinct mRNAs reflecting the presence or absence of 2 alternatively spliced exons (Figure 1.4). Splice I is a separate exon that codes for 17 amino acids, including 5 serine and 1 threonine residues (sites of potential protein phosphorylation), and is inserted between the proline-glutamine rich region and the zinc-finger domain. The second splice is an alternative 5' splice junction that results in the insertion of 3 amino acids (1 serine and 1 threonine) between fingers 3 and 4 and which affects the DNA-binding specificity of the protein (Bickmore *et al.*, 1992). Using RNase protection it has been shown that the most common transcript in human and mouse kidneys is that including both extra splices, whereas the least abundant is that with neither splice (Haber *et al.*, 1991). The relative distribution of these four transcripts has also been examined and found to be conserved between normal fetal kidney and cases of WT with intact transcripts. In the developing mouse kidney, the ratio of the 4 splice variants remained constant with time and was also similar in the genital tissue.

### *1.4.2 Genetics of* WT1

Although substantial evidence had been accumulated to support a role for *WT1* in the formation of WT, direct proof awaited the demonstration in the tumour of mutations involving only this gene. In several cases, deletions have been reported that include *WT1* but that extend beyond the locus and could involve other genes (Lewis *et al.*, 1988; Gessler *et al.*, 1990). Using Southern blot analysis, gross rearrangements of the gene have been detected in approximately 8% of tumours, and homozygous deletion within *WT1* demonstrated (Cowell *et al.*, 1991; Tadokoro *et al.*, 1992). In accordance with Knudson's 'two-hit' hypothesis, a bilateral case has been described with an intragenic germline deletion that had become homozygous in both tumours (Huff *et al.*, 1991). Most mutations are not

detectable at this level, however, and the reverse transcriptase-polymerase chain reaction (PCR) method has been employed to amplify and analyse mRNA from a collection of tumours. Using this technique, a WAGR patient has been found with a constitutional deletion of 11p13 and an intragenic deletion of the remaining *WT1* allele (Brown *et al.*, 1992). It is of interest that several genes flanking *WT1* have been shown to be expressed in fetal kidney (Bonetta *et al.*, 1990), but there is no genetic evidence to support a role for these in WT.

In addition to homozygous inactivation of *WT1*, there may be another mechanism that contributes to the formation of the tumour. A case of a sporadic tumour has been described where equal amounts of the wild-type and mutant *WT1* transcripts are expressed (Haber *et al.*, 1990). This mutation results in a protein that lacks one of the zinc fingers, and which if functional, could suppress the activity of the normal protein. In a separate study, chemical mismatch cleavage analysis has been employed to examine the zinc-finger region of *WT1* for genomic point mutations (Little *et al.*, 1992). Two exonic single base changes were detected: the first involved a nonsense mutation leading to an arginine residue becoming a stop codon in zinc finger 3, while the second was a missense mutation in one allele resulting in a change of an arginine to a cysteine in zinc finger 2. By comparison to the structure of EGR-1, the mutation in the second example seems to be critical for DNA-binding. Furthermore, by analogy to *hunchback*, a *Drosophila* transcription factor (Tautz *et al.*, 1987), the resulting cysteine may confer a different binding specificity for WT1 and this might well disrupt or compete with the binding activity of the normal allele. Such 'dominant-negatives' have been found for p53, a tumour suppressor gene implicated in many human malignancies (Levine *et al.*, 1991), and indicate that a single genetic hit at the *WT1* locus may be sufficient for tumourigenesis in certain cases. Another, equally probable mechanism is that inactivation of one *WT1* allele could be interacting with alterations at a second locus. There is evidence that both the 11p13 and 11p15 loci can contribute to the formation of a single tumour (Henry *et al.*, 1989).

The isolation of *WT1* has allowed the involvement of the 11p13 locus in genital abnormalities to be investigated. Two children presenting with WT, hypospadias and cryptorchidism were shown to have constitutional null mutations in *WT1* and these cases raise the possibility that reduced dosage of the gene can lead to malformations of the genitalia (Pelletier *et al.*, 1991b). One of these cases also indicated that *WT1* may have a role in familial WT. In two independent studies, patients with Denys-Drash syndrome, that characteristically have more severe genital abnormalities than those with WAGR, were examined (Pelletier *et al.*, 1991c; Bruening *et al.*, 1992). Germline point mutations in the zinc-finger domain of one allele of *WT1* were found in all cases, except one that affected alternative splicing, and suggests that altering the DNA-binding specificity of the protein may lead to a greater effect on genital development than its loss.

### *1.4.3 The pattern of* WT1 *expression*

The expression of *WT1* has been examined in both the tumour and human embryonic tissue by northern blotting and *in situ* mRNA hybridisation. It has been shown by northern analysis that WT1 detects a transcript of approximately 3 kb in baboon kidney and spleen (Call *et al.*, 1990). In another study human fetal tissues were examined and a 3.2 kb transcript was observed in the kidney, spleen, testis and ovary with an additional 2.7 kb transcript in the testis (Pritchard-Jones *et al.*, 1990). Very weak expression was also found in the brain, but none was observed in the heart, skin, adrenals, stomach, liver, eye or muscle. The pattern of *WT1* expression in an 18 week human fetal kidney was examined using *in situ* mRNA hybridisation (Pritchard-Jones *et al.*, 1990). There was a low level of labelling in the condensed mesenchymal cells, which increased in the renal vesicle and was then restricted to the maturing renal corpuscle. It was strongest within the podocyte cells of Bowman's capsule with no expression being observed in the rest of the nephron. A similar but much weaker pattern was seen in the normal kidney adjacent to a WT from a 10 month old male. To investigate a possible role in early development, human embryos (6-7 weeks)

were examined and the metanephric mesenchyme and the glomerular epithelium of the mesonephros were found to be positive. In addition, the germinal epithelium and the sex cords of the gonads, together with the mesothelial lining of the coelomic cavity and its contents, expressed *WT1*.

Tumours have also been examined using northern analysis and, although the 3.2 kb transcript was detected in every case, there was a 500-fold variation in the level of *WT1* expression, with tumours that were predominately blastemal or epithelial having the highest level and stromal ones the lowest (Pritchard-Jones *et al.*, 1990; Huang *et al.*, 1990; Miwa *et al.*, 1992a). *In situ* mRNA hybridisation was used to show that *WT1* expression was dependent on the histology of an individual tumour (Pritchard-Jones & Fleming, 1991). The blastemal areas were found to express the gene, whereas the stromal elements did not label above the background level. The highest level of positive signal was associated with epithelial differentiation and in particular with the glomeruloid-bodies. The majority of tubular structures also labelled, but, in the few cases that did not express *WT1*, immunohistochemistry demonstrated that these either represented elements that had developed to a stage beyond the S-shaped body or were derived from the ureteric bud. *WT1* transcripts are therefore found in the malignant equivalent of cells that express the gene during normal nephrogenesis.

As *WT1* was isolated from an haematopoietic cell line, it is of interest that northern analysis has demonstrated expression of the gene in cell lines from human leukaemias (Pritchard-Jones & Hastie, 1990; Miwa *et al.*, 1992b). *WT1* was not, however, transcribed in all cases of the disease: those of immature phenotype such as acute lymphoblastic leukaemia and acute mylogenous leukaemia expressed the gene, while those of a more mature type, including chronic lymphocytic leukaemia, did not (Miwa *et al.*, 1992b). This pattern, together with expression in the spleen, suggests that *WT1* may be involved in the early stages of haematological cell differentiation.

### *1.4.4 Interaction of* WT1 *with other genes*

The expression pattern of the *WT1* gene is consistent with its having a role in normal development, and there has been some discussion of the possible target sequences for this transcription factor. *WT1* could function by repressing genes involved in cell proliferation or alternatively by activating those required for differentiation. Possible targets that fit into the first category include the proto-oncogene *N-myc* and the mitogen *IGF-II*, both of which are expressed in the metanephric mesenchyme at a level that decreases as epithelial differentiation takes place (Mugrauer *et al.*, 1988; Mugrauer & Ekblom, 1991; Brice *et al.*, 1989). These two genes are also expressed at a high level in WT: *N-myc* is transcribed in the tumour at a much higher level than in the embryonic kidney (Nisen *et al.*, 1986), while that of *IGF-II* is comparable to embryonic kidney (Reeve *et al.*, 1985; Scott *et al.*, 1985). *IGF-II* is of particular interest because of the association of this gene with the 11p15 locus (for a review of the *IGF-II* gene, see Schofield, 1991). In a recent study, WT1 was co-transfected into cultured cells with the promoter region of the two *IGF-II* transcripts expressed in human fetal kidney and the basal level of *IGF-II* was found to be significantly repressed (Drummond *et al.*, 1991; Drummond *et al.*, 1992). Furthermore, examination of the *IGF-II* promoter sequence revealed several potential high affinity binding sites for WT1 and gel shift assays have confirmed this. As to the second category, any of the genes that are expressed as the stem cells of the metanephric mesenchyme condense and undergo epithelialisation can be considered as possible targets for WT1 (for details see section 1.1.4).

Many developmentally important genes are expressed in a specific way in the kidney as it forms and any of these could be regulated by WT1. Several of the *Hox* genes are expressed during the development of the mesonephros and metanephros (reviewed by Holland & Hogan, 1988). *In situ* hybridisation studies have shown that while several of the genes from this family are expressed uniformly in the metanephric mesenchyme and the tubular epithelium (Dolle & Duboule, 1989; Gailliot *et al.*, 1989), others are transcribed at a higher level in the forming nephron (Dolle & Duboule, 1989) or restricted to the collecting

system (Kress *et al.*, 1990). *HNF1* and *vHNF1* are two genes that contain a homeodomain with an extra 21 amino acids. *HNF1* was first identified as a transcriptional regulator of several liver specific genes and may form heterodimers with *vHNF1* (Chouard *et al.*, 1990; Rey-Campos *et al.*, 1991). In the rat embryonic kidney *vHNF1* is expressed in metanephric mesenchyme undergoing the initial aggregation after induction and in conjunction with *HNF1* in later stages of nephrogenesis (Lazzaro *et al.*, 1992).

The murine *Pax* genes are a family which have a common protein binding domain, the paired-box, which was first described in the *Drosophila* segmentation genes *paired* and *gooseberry* (Bopp *et al.*, 1986) and is conserved in man (Burri *et al.*, 1989). In most members of this family, a second domain, the paired homeodomain that has diverged from the homeobox, is also observed. The *Pax* genes are expressed in a tissue specific manner during development with *in situ* mRNA hybridisation data showing that *Pax-2* and *Pax-8* which show extensive homology are expressed during the development of both the mesonephros and the metanephros. *Pax-2* is expressed in the ureter, the condensed mesenchyme and epithelial derivatives of the metanephros and is down-regulated as the nephron continues to differentiate (Dressler *et al.*, 1990), while the expression of *Pax-8* is similar but does not include the ureter (Plachov *et al.*, 1990). The localisation of *Pax-2* expression in WT has been examined using polyclonal antibodies and shown to be restricted to areas of epithelial differentiation (Dressler & Douglass, 1992).

In contrast to these surmised targets, a direct analysis has been carried out on the binding of the WT1 protein to DNA. Using a construct containing the zinc-finger region of WT1, it has been shown that this domain identifies binding sites that are similar to those recognised by *EGR-1* (Rauscher *et al.*, 1990). In the same study, the alternative splice of 3 amino acids inserted between the 3rd and 4th zinc finger reduced binding affinity, as did a mutation that had previously be described in a case of WT where the 3rd zinc finger was deleted (Haber *et al.*, 1990). The glutamine-proline rich region has been observed to repress transcription when bound to the EGR-1 site (Madden *et al.*, 1991). The most prevalent mRNA species is, however, the one that contains the extra 3 amino acids (Haber

*et al.*, 1991) and this form has recently been shown to encode a protein that can also bind with a different specificity to DNA (Bickmore *et al.*, 1992). This observation has led to the suggestion that the two alternative forms recognise distinct binding sites and may have different functional roles. Furthermore, the germline point mutation described in a case of Denys-Drash syndrome that affected alternative splicing supports the idea that the *WT1* isoforms may play different roles during the development of the urogenital system (Bruening *et al.*, 1992).

## 1.5 The murine homologue of *WT1*

Initial experiments using the human *WT1* gene to probe northern blots had indicated that the gene was expressed in the mouse fetal kidney and this led to it being used to screen a 17 day mouse embryonic kidney cDNA library (Buckler *et al.*, 1991). Five clones were isolated, but none of these included the part corresponding to the 5' end of the characterised human cDNAs, so an adult mouse testis library was subsequently screened to obtain longer clones. The complete nucleotide sequence of the murine WTI cDNA (3,089 bp) is given in the appendix. If the mouse and human polypeptides are compared, a high degree of homology is seen (>96%) with the differences occurring mainly outside the zinc-finger region, although these are not conservative changes (see appendix for details). The expression pattern of this gene is not considered here, but will be described in chapter 3.

The occurrence of WT in species other than man is very rare and almost unheard of in the mouse (Hard, 1984a,b). It should also be born in mind that the Dickie's *Small-eye* (*Sey*$^{dey}$) mouse mutant carries a deletion of part of chromosome 2, the murine homologue of the WAGR region of human chromosome 11, that includes both the *AN2* and *WT1* loci (Glaser *et al.*, 1990). The homozygotes die before implantation, while the heterozygotes are born with eye abnormalities that vary in severity and a reduction in body size of about 10%, but have no evidence of kidney abnormalities or of WT (Theiler *et al.*, 1978). In addition to

this spontaneous variant, two further allelic mutations have been described; *Sey*$^{H}$ (Lyon & Searle, 1979) and *Sey* (Hogan *et al.*, 1986). It has been suggested that *Sey*$^{Dey}$ and *Sey*$^{H}$ are caused by large deletions, whereas *Sey,* that has a much less extreme phenotype, is an intragenic deletion or point mutation. That a mouse carrying a deletion of one *WT1* allele never develops WT could reflect differences in the degree of genetic complexity between the two species or alternatively those involved in nephrogenesis.

The mouse, nevertheless, is an excellent system in which to study *WT1* and offers several distinct advantages; its greater developmental and genetic accessibility, the production of transgenic animals and the *in vitro* kidney culture system. The cloning of the murine homologue of the *WT1* gene should allow such advantages to be exploited.

## 1.6 Thesis outline

In this introduction, the current understanding of both the genetic and developmental basis of WT has been laid out so that the implications of the results obtained here, describing the expression pattern of the mouse *WT1* gene during development, can be considered. The next chapter, details the materials and methods used during the course of this work. The results are presented in three separate chapters, each with its own local introduction and short discussion. The first of these examines the expression of *WT1* in the developing mouse embryo using *in situ* mRNA hybridisation, the second details its expression in the metanephric kidney both *in vivo* and *in vitro* with the final chapter investigating a model that had been proposed to give tumours, that have a morphology similar to WT, in the mouse. Finally, in the last chapter, an overview of the thesis will discuss the significance of this data and suggest work that will be relevant in the further understanding of the role of *WT1*.

# CHAPTER 2

# MATERIALS AND METHODS

In the course of this thesis a wide variety of techniques were used and these are outlined in this chapter. A list of the companies that supplied the chemicals, antibodies and enzymes is given at the end in Section 2.8.

## 2.1 Collection of embryonic material

A breeding colony of Swiss mice, an albino outbred strain, was maintained by the animal house at the Western General Hospital. Timed matings were set up, so that the morning on which the vaginal plug was noted was designated as day 0.5 post coitum (p.c.). Pregnant females were killed by cervical dislocation and their uterine horns removed under sterile conditions. After isolation, the embryos were staged accurately using the morphological criteria given by Theiler (1989), and processed as described below in Section 2.2. The 15 and 19 day p.c. embryos had their heads removed, with the older stage being skinned prior to processing. Metanephric kidneys were also dissected out in Dulbecco's phosphate buffered saline (PBS) with disposable hypodermic needles, using a Wild-Heerbrugg M7A dissecting microscope.

## 2.2 Processing of tissue samples

The method chosen for fixing and embedding a particular tissue depended on the subsequent procedures that would be applied and a detailed explanation of these considerations is given in Bancroft and Stevens (1990).

### *2.2.1 Paraffin wax embedding*

For *in situ* mRNA hybridisation, tissue was fixed overnight at 4$^{o}$C using freshly prepared 4% paraformaldehyde (PFA) in PBS and for immunohistochemistry, 10% neutral

buffered formalin was employed (50 ml formaldehyde, 9.3 g $NaH_2PO_4$, 2.1 g NaOH and 450 ml $H_2O$). After fixation, the specimen was washed in PBS for 30 min, transferred to 30% EtOH for 30 min and then into 70% EtOH for 30 min. The tissue could be left at this stage indefinitely, as long as the EtOH was changed occasionally, but was usually left overnight. After a third change of 70% EtOH, dehydration was completed using 100% EtOH (3 steps of 1 h). The specimen was cleared with xylene (2 changes of approximately 15 min), placed in a 60°C oven for 10 min and then transferred to molten paraffin wax (melting point 56°C). After three 1 h changes, the tissue was finally embedded in fresh wax using copper embedding irons as moulds or in glass dishes for embryos less than 10 days old.

The tissue was sectioned at a thickness of 7 µm using a Reichert-Jung 2030 microtome. For *in situ* mRNA hybridisation, 3 strings were mounted in parallel under sterile conditions on slides coated with 3-aminopropyltriethoxy-silane (TESPA) using a floating-out bath and dried overnight at 60°C. Strings for immunohistochemistry were cut into individual sections, mounted on welled slides (C.A. Hendley, Ltd.) using a hot plate and dried overnight at 50°C. Routine histological analysis could be carried out on either type of section after staining with haematoxylin and eosin (H & E, see Bancroft and Stevens, 1990 for details). The slides for *in situ* mRNA hybridisation were stored with desiccant until required.

### *2.2.2 Araldite embedding for electron microscopy*

The tissue was cut into small pieces (approximately 1 $mm^3$) and fixed overnight at room temperature in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3, 468 mOsm) containing 0.1 M sucrose. After fixation, the specimen was rinsed in the buffer (2 times 10 min) and post-fixed in buffer containing 1% osmium tetroxide for 1 h. It was then rinsed and dehydrated through the following series of graded alcohols: 10, 30, 50, 70, 95 and 100% EtOH. The tissue was cleared using propylene oxide (2 times 10 min), before being transferred to a foil dish containing a thin layer of araldite CY212 and left overnight at room temperature to allow the resin to infiltrate. The dish was then heated to 60°C for 35

min and the specimen transferred to fresh araldite, that was then polymerised by heating at 60°C for 3 days.

All of the cutting was carried out by the EM department, with a LKB5 ultramicrotome using glass knives. Thin sections (70 nm) were floated out onto distilled $H_2O$, picked up using 3 mm copper grids (TAAB) and stained in saturated uranyl acetate in 50% EtOH at room temp for 30 min. After 10 s washes in 50%, 30% and 10% EtOH followed by distilled $H_2O$, the sections were stained in 1% Reynold's lead citrate for 15 min. Finally the sections were rinsed in $dH_2O$ and allowed to air dry. A Philips 300 transmission electron microscope (TEM) was used to view and photograph the sections.

## 2.3 Preparation of DNA

The production of the riboprobe described in section 2.4.1 requires a clean DNA sample as a template and this was obtained using the techniques detailed below (general reference; Sambrook *et al.*, 1989).

### *2.3.1 Bacterial strain and plasmid vector used*

The WT1 insert (kindly provided by Prof. David Housman; sequence given in the appendix) had been cloned into the Eco RI sites of pbluescriptKS+ by Wendy Bickmore. This 3 kb vector was derived from the pUC 19 plasmid (Messing, 1983) with the addition of T3 and T7 promoter sequences that flank the polylinker, thus enabling the production of both sense and anti-sense mRNA transcripts. The advantages of this vector include; the high copy number, the ampicillin resistance gene and that the artificial polylinker has been inserted into the β-galactosidase gene (lac-Z) as a test for recombinant plasmids. The plasmid was then transformed into the XL-1 Blue bacterial strain and given the clone name pkS/2.

### *2.3.2 Isolation of plasmid DNA*

To isolate plasmid DNA from bacterial culture, a protocol based on the alkaline lysis method of Birnboim and Doly (1979) was employed. A single colony was used to inoculate 500 ml of terrific broth [13.3 g bacto-tryptone, 26.6 g bacto-yeast extract and 4.4 ml glycerol per l with 1/10 volumes of 1 M phosphate salts (164.32 g $K_2HPO_4$, 23.1 g $KH_2PO_4$) added prior to use] containing ampicillin, that was then grown up overnight on a shaker at 37°C. The cells were harvested by spinning at 8,000 rpm for 10 min, the supernatant poured off and the pellet resuspended in GTE [50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA)] to give a final volume of 18 ml. A further 2 ml of GTE containing 10 mg/ml of lysosyme was added and left for 10 min at room temperature or until the solution became viscous. To denature the chromosomal DNA, 40 ml of freshly prepared 0.2 M NaOH, 1% sodium dodecyl sulphate (SDS) was added and the solution kept on ice for 5 min. To precipitate the bacterial debris, 20 ml of cold 5 M potassium acetate (pH 4.8) was then added and after 15 min on ice the solution was spun at 15,000 rpm (15 min). The supernatant was separated by filtering through muslin and the plasmid DNA precipitated by the addition of 0.6 volumes of isopropanol. After 1 h at room temperature it was spun at 8,000 rpm for 15 min. The supernatant was discarded, the pellet rinsed with 70% EtOH and allowed to air dry before being resuspended in 4 mls of TE (10 mM Tris, 1 mM EDTA, pH 8.0).

The volume was measured accurately and designated as X, with 0.1 ml/X ml of 10 mg/ml EtBr and X g of CsCl being added. This was then put into ultracentrifuge tubes, balanced to within 0.1 g, sealed with a tube sealer and spun overnight at 100,000 rpm in a Beckman TL-100 ultracentrifuge. Taking care not to disturb the gradient, the lower of the 2 bands observed was removed using a pastette and put into fresh tubes. These were topped up with the EtBr/CsCl solution and respun. The EtBr was completely removed using water saturated butan-1-ol and the DNA precipitated by the addition of EtOH (placed at -20°C for 30 min and then centrifuged to pellet the DNA) before being redissolved in TE. The final

concentration was determined by reading the optical density using a spectrophotometer (Cecil CE594).

### *2.3.3 Digestion of DNA*

In order to transcribe RNA, the template DNA had first to be linearised using the appropriate restriction enzyme. An enzyme was chosen that cleaved at a site in the polylinker sequence downstream of the insert with respect of the RNA polymerase promoter, but did not cut within the sequence. In the case of pkS/2, Bam HI was used in accordance with the manufactures instructions for the T3 promoter and Hind III for T7. After linearisation, the plasmid DNA was purified by phenol/chloroform extraction and isolated using EtOH precipitation [1/10 volumes of 3 M NaOAc and 3 volumes of cold EtOH (-20$^{o}$C for 30 min)].

### *2.3.4 Agarose gel electrophoresis of DNA fragments*

Mini gels were routinely used to check that enzymatic digestion was complete and that the plasmid was properly linearised (a single sharp band). In order to prevent overheating of the mini-gel equipment 0.5 x TBE buffer (10 x TBE: 1 M Tris-HCl, 0.8 M Boric Acid, 20 mM EDTA, pH 8.3) was used to prepare 1% agarose gels. Stop mix (0.2 M EDTA, pH 8.1, 15% ficoll and Orange G) was added to the DNA and this migrates ahead of the fragments, thus acting as a visible migration front. The gels were run at 75 volts for approximately 30 min, stained with EtBr and viewed under UV light, before being photographed. Lambda DNA digested with Hind III was used as a size marker.

## 2.4 *In situ* mRNA hybridisation

The expression pattern of a gene can be visualised using the technique of *in situ* mRNA hybridisation on sections of tissue. The protocol outlined below was developed by

Duncan Davidson and Liz Graham (Davidson *et al.*, 1988) from the method of Wilkinson *et al.* (1987a, b).

### *2.4.1 Preparation of riboprobe*

Single-stranded sense and anti-sense RNA probes were prepared, that incorporated $^{35}$S-UTP, using T7 and T3 polymerase respectively. The transcription reaction was carried out at 37$^{o}$C for 50 min with fresh polymerase being added after 25 min. A concentration of 50 mM dithiothreitol (DTT) was maintained at all times to prevent oxidation of the radiolabel. To estimate the incorporation of radiolabel, a 1 µl aliquot of the reaction mix was added to 19 µl of TE and divided between two glass fibre filters (Whatman GF/A filters). One was airdried, while the other was washed twice with cold TCA and once with EtOH. After drying, the filters were placed in pots containing 10 ml of Aquasol and the specific activity estimated in a scintillation counter (Packhard 1500) standardised for $^{35}$S emissions. The DNA template was removed by DNase I digestion (37$^{o}$C for 10 min) and the reaction stopped using 100 mM EDTA. The volume was increased with TE (pH 7.4) so that the RNA could be extracted using phenol/chloroform. The probe was EtOH precipitated at -20$^{o}$C overnight to remove any unincorporated nucleotides before being pelleted and washed twice with 80% EtOH and once with 100% EtOH. The pellet was dried in a vacuum and redissolved in water containing 0.1% Diethyl pyrocarbonate (DEPC). As cells are less accessible to transcripts of more than 150 bp, alkaline digestion (80 mM $NaHCO_3$, 120 mM $Na_2CO_3$, pH 10.2, at 60$^{o}$C) was used to generate random fragments of this length. The time required to produce transcripts of approximately 150 bp was calculated using the following equation, with 60 min being an absolute maximum;

$$t(min) = \frac{lo - 0.1}{0.011 \times lo}$$

where lo = insert size in kb

The probe was recovered by EtOH precipitation (minimum of 3 h at -20°C), spun, washed and dried. The pellet was redissolved in TE (pH 7.4) and the final specific activity estimated as described previously. At this stage the probe was stored at -70°C until required.

### *2.4.2 Treatment of slides prior to hybridisation*

The slides were dewaxed in two 10 minute changes of xylene and then rinsed in 100% EtOH to remove the xylene (2 steps of 2 min). A series of graded alcohols was used to rehydrate the sections (90, 70, 50 and 30% EtOH) which were then washed in 0.85% NaCl and PBS for 5 min at a time. The tissue was fixed by being immersed in 4% PFA for 20 min. After a further two 5 minute washes in PBS, the slides were treated with Protinase K (20 µg/ml in 50 mM Tris, 5 mM EDTA, pH 7.2) that digests the surface of the tissue, thus improving the access of the probe to the cellular mRNA. The time used was reduced from the standard 7.5 min to 3 min for delicate samples such as the kidney cultures. The slides were then rinsed in PBS for 5 min and fixed in 4% PFA for 5 min, before being treated with acetic anhydride (2.5 ml per 1000ml of 0.1 M Triethanolamine, pH 8.0). This neutralises any basic charges on the sections that would bind the probe non-specifically. The slides were rinsed in PBS and 0.85% NaCl before being dehydrated through the graded alcohols. The slides were allowed to air dry and stored in a desiccator before use, which was usually within 24 h, but could be up to 4 months.

### *2.4.3 Hybridisation*

The probe was diluted in hybridisation mix [50% formamide, 10% dextran sulphate, 1 x Denhardt's solution (50 x solution: 5 g ficoll, 5 g polyvinylpyrrolidone, 5 g BSA), 20 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 5 mM EDTA, 10 mM $NaH_2PO_4$, 0.5 mg/ml yeast RNA and 50 mM DTT added just prior to use] to give an estimated 1.2 x $10^5$ dpm/µl. The diluted probe was denatured by heating it for 2 min at 80°C before cooling rapidly on ice.

A 10 µl aliquot of probe was placed on each string of sections, with sense probe being routinely placed on the middle string and anti-sense on the outer ones. Siliconised

coverslips were placed on top of each string to prevent the sections from drying out. The slides were placed in a slide box containing a cloth soaked in 50% formamide, 5 x sodium saline citrate (20 x SSC: 175.3 g NaCl, 88.2 g tri-sodium citrate) and this was then sealed and submerged in a waterbath at 55°C for 16-18 h.

### *2.4.4 Post-hybridisation washes*

The slides were removed from the box and put into 5 x SSC, 10 mM DTT at 55°C for between 15 to 30 min, or until all the coverslips were removed, before being transferred to an high stringency wash of 50% formamide, 2 x SSC, 0.1 M DTT at 68°C for 20 min. The sections were treated with RNaseA to remove any non-specifically bound probe and the slides had to first be washed with NTE (0.5 M NaCl, 10 mM Tris, 5 mM EDTA, pH 7.5) in order to remove any traces of formamide. Thus, after three 10 min NTE washes at 37°C, the slides were incubated with 40 mg/ml RNase A in NTE for 30 min. After a further 15 min wash in NTE, the slides were returned to a high stringency wash. The slides were then washed in 2 x SSC (3 steps of 10 min) and similarly in 0.1 x SSC. They were finally dehydrated through graded alcohols, which each contained 0.3 M ammonium acetate, and air dried in a dust-free environment.

### *2.4.5 Autoradiography of slides*

The slides were coated under safelight (S 902) in Ilford K5 gel emulsion that had been warmed to 40°C and then diluted 1:1 with distilled water. They were left to dry in a light tight box overnight and then placed with in a sealed box with desiccant at 4°C for between 2-4 weeks.

### *2.4.6 Developing the slides*

Under safelight the slides were developed for 4 min using D19, rinsed for 10 seconds in distilled water and then fixed for a further 4 min in a 1:3 solution of kodafix in distilled water. After a further two 10 minute washes in water, the slides were stained in 1%

Methyl Green and air dried. They were mounted in DPX and examined and photographed using a camera mounted on either a Zeiss Universal microscope or a Wild M400 dissection photomicroscope.

### *2.4.7 Production of digitised images*

The inherent problems associated with the photography of the data from *in situ* mRNA hybridisation experiments led to the development of a computer-aided technique that superimposes the bright and dark field images. This was used in conjunction with conventional photography in the production of the figures in Chapters 3 and 4.

A detailed description of the technique is given in Monaghan *et al.* (1991), but a brief summary is given here. The image was projected onto a monitor using a video camera mounted on the microscope. A software package was used to grab (digitise) the image which involved converting the image into a format that the computer could read and then display on the computer screen. This was repeated for both the bright and dark field images which were merged and thresholded to give a true signal using a shellscript program written under the Unix operating system. The image could then be photographed directly from the screen. All the digitising was carried by Liz Graham.

## 2.5 Culture of kidney rudiments

The whole-organ culture material used during this project was kindly supplied by Jonathan Bard and the transfilter tissue by Prof. Lauri Saxen. The embedding and sectioning was carried out by Allyson Ross.

Metanephric kidney rudiments were dissected from 11-11.5 day embryos with hypodermic needles and trimmed of surplus non-metanephric mesenchyme in serum-free Dulbecco's modification of Eagle's medium (DMEM) medium (11 day rudiments have a simple ureteric bud extending into the metanephric mesenchyme which is uninduced; by

11.5 day, this bud has bifurcated and the mesenchyme has been induced in the sense that, when isolated, it will differentiate in the absence of further inducing agent). The rudiments were picked up in a drawn-out Pasteur pipette and transferred to sterile Millipore discs (pore size 0.45 μm, diameter ~2 mm) or small squares of Nuclepore filter (pore size 1.0 μm) on a trowell-type, stainless steel screen (some of whose holes had been enlarged). The screen had already been placed in a 3.5 cm petri dish to which had been added sufficient medium to ensure that the rudiments were held in place by surface tension at the air-medium interface.

The rudiments were cultured in DMEM to which was added sodium bicarbonate (1.5 g/l), glutamine (0.3 g/l) and 10% foetal calf serum. This medium is not standard as it has one third the amount of usual bicarbonate: DMEM is normally used to culture rapidly growing cells which make acid; the amount of kidney tissue is so small that little acid is produced and normal DMEM becomes very alkaline. The rudiments were cultured for up to 6 days at 37$^{o}$C in 7% $CO_2$ with the medium being changed after 3 days. Additional experiments were carried out with media that contained LIF (leukaemia inhibitory factor) or cytochalasin B for 6 days. In the case of LIF, Esgro was added to the media (8000 U/ml and 4000 U/ml were used). For the experiments involving cytochalasin B, 0.2 μg/ml was added.

For the transfilter cultures, mesenchymes were separated from their ureters with fine needles after the kidney rudiments had been incubated with trypsin (1%) and pancreatin (0.5%) in PBS at 0$^{o}$C for 90 s and allowed to recover for 10 min at room temperature. Meanwhile, neural tube was dissected from the 11 day embryos and cut into small (~1 mm) pieces. To prepare the culture, the squares of Nuclepore filter were placed over the holes in a trowell grid in a 3.5 cm petri dish containing medium and fragments of neural tube placed on them. These were then immobilised with a tiny drop of molten agar in PBS which was allowed to solidify at 4$^{o}$C. After about 5 min, the filter was turned over and 2 or 3 mesenchymes placed on the filter over the neural tube and these too were immobilised with agar. After the second agar drops had solidified, the dishes were incubated routinely for 1-4 days.

The cultured tissue was fixed in 4% PFA, embedded in wax, sectioned at 5 μm and mounted on TESPA coated slides as described previously (Bard and Ross, 1991).

## 2.6 Production of pseudo Wilms' tumours

The data presented in Chapter 5 was obtained using the method described below, which was based on one first described by Javadpour and Bush (1972), with the addition of the adult kidney as a host site.

### *2.6.1 Collection of embryonic kidneys*

Breeding colonies of Swiss, CBA and 129/Sv mice strains were maintained by the animal house and timed matings set up. The metanephric rudiments from embryos of 12.5, 13.5 and 14.5 days p.c. were isolated as described in section 2.1 and stored for up to 2 h on ice.

### *2.6.2 Operating procedures*

Sexually mature mice were anaesthetised using an intraperitoneal injection of Avertin (0.12 ml Avertin made up to 10 ml with PBS and used at a dosage of 0.2 ml/10g body weight). For the operations involving the testis, male animals were laid on their backs and the lower abdomen swabbed with alcohol. Using sterile instruments an incision was made in both the skin and the muscle wall and the animal's left testis exposed, with a small tear being made in the capsule. A whole embryonic kidney (14.5 day rudiments were cut in half) was sucked into a drawn-out Pasteur pipette, the tip of which was then inserted through the tear. The rudiment was expelled at a distance of approximately 5 mm from the tear. In the case of the kidney as the host site, male and female mice were laid on their front, the area below the ribs swabbed and an incision made parallel to the backbone exposing the animal's right kidney. The wound was stitched with surgical suture (Ethicon) and the skin

held together with a metal wound clip. For both types of operation the contralateral organ was left untreated and severed as the control. The animals were kept warm until fully recovered and then monitored for any signs of ill health for the duration of the experiment.

### *2.6.3 Recovery of implanted tissue*

The animals were killed by cervical dislocation after a period of between 4 and 26 weeks with both the operated and control organs being removed and the peritoneal cavity examined for metastases. For the kidney, the implanted tissue was usually visible and could be removed together with a small area of the adjacent adult organ. The tissue proved more difficult to locate in the case of the testis and usually required the whole adult organ to be processed into wax and then serially sectioned. The contralateral controls were examined for superficial changes and a random selection were fixed and embedded in wax.

The implanted tissue was processed for *in situ* mRNA hybridisation, immunohistochemistry and TEM as described previously. In almost every case, several sections were stained with H & E for routine histological analysis and a selection of these was then photographed (see section 2.4.7).

## 2.7 Immunohistochemistry

The indirect immunohistochemistry method was employed, which involves using an unlabelled primary antibody that recognises and binds to the tissue antigen. This is detected by an enzyme-labelled second antibody raised in another species and a coloured reaction product is produced using a substrate of the enzyme. A primary antibody anti-α tubulin, that recognised a constituent present in all cells, was used as a positive control and omission of the primary antibody served as the negative control. In addition to brush border antibody (BB110; Miettinen & Linder, 1976; Ekblom *et al.*, 1980a), a novel monoclonal antibody, WT1.27, was used (kindly provided by Veronica van Heyningen and Jonathan Bard). This

had been raised in BALB/c mice by immunising them with a sonicated suspension of cryopreserved human WT (van Heyningen *et al.*, 1982) and screening was carried out on human and mouse embryonic kidney sections.

The tissue sections were dewaxed by immersion in xylene and rehydrated through graded alcohols, before being washed under running tap water. Where necessary, sections were treated with Protinase K, before being rinsed in PBS (2 times 5 min). To block non-specific binding of the primary antibody, whole serum (diluted 1:10 with PBS) was placed on the sections for 10 min. The first antibody was put on at the appropriate dilution (with PBS) for 60 min at room temp or overnight at 4$^{o}$C. To remove any traces of the first antibody, the slides were washed twice with PBS and the serum blocking step repeated, before the second antibody (alkaline phosphatase conjugated anti-mouse for WT1.27 and anti-rabbit for BB110, both used at a concentration of 1:200 in PBS) was put on for 60 min at room temperature. After 2 further PBS washes, the slides were flooded with 0.1 M Tris-HCl (pH 9.5) for 5 min. The substrate was prepared using the Vector Black kit II as directed by the manufacturers, with endogenous alkaline phosphatase activity being blocked by adding levamisole (2 mM) to the substrate solution. Sections were immersed in the substrate for up to 20 min and for more intense staining allowed to develop in the dark. The reaction was stopped in running water and the sections counter stained with 0.01% Malachite green, before being air dried, mounted in DPX and photographed using a Zeiss Universal microscope.

## 2.8 List of suppliers

| | |
|---|---|
| **Agar Scientific** | Araldite CY212 |
| **Amersham** | $^{35}$S-UTP |
| **BCL** | CsCl |

| | | |
|---|---|---|
| **BDH Chemicals** | Ammonium acetate | $K_2HPO_4$ |
| | Boric acid | Isopropanol |
| | Butan-1-ol | NaCl |
| | Chloroform | NaOH |
| | DPX | Paraffin wax |
| | Formaldehyde | PFA |
| | Formamide | Potassium acetate |
| | Glucose | Propylene oxide |
| | Glycerol | SDS |
| | Harris's haematoxylin | Sucrose |
| | HCl | Uranyl acetate |
| | $KH_2PO_4$ | Xylene |
| **Boehringer Mannheim** | DTT | RNaseA |
| | Protinase K | Tris |
| | Restriction enzymes | |
| **Dupont** | Aquasol | |
| **Difco** | Bacto-tryptone | Bacto-yeast extract |
| **Gibco** | Ultra pure phenol | |
| **Kodak** | D19 | Kodafix |
| | Ilford K5 gel emulsion | |
| **Oxkem** | Osmium tetroxide | |
| **Oxoid** | PBS | |
| **SAPU** | Sheep serum | |
| **Sigma** | Acetic anhydride | Lysosyme |
| | Ampicillan | Methyl green |
| | Anti-$\alpha$-tubulin | $NaHCO_3/Na_2CO_3$ |
| | BSA | Orange G |
| | DEPC | Pancreatin |
| | Dextran sulphate | Polyvinylpyrrolidone |
| | EDTA | Triethanolanine |
| | EtBr | TESPA |
| | Ficoll | Trypsin |
| | Levamisole | Yeast RNA |
| **Stratagene** | DNase I | T3 polymerase |
| | pbluescriptKS+ | T7 polymerase |
| **TAAB** | Eosin | Sodium cacodylate |
| | Glutaraldehyde | |
| **Vector Labs** | Vector Black kit II | |

# CHAPTER 3

## THE EXPRESSION OF *WT1* DURING MOUSE EMBRYOGENESIS

## 3.1 Introduction

The expression pattern of the *WT1* gene during human embryonic development has been studied using both northern blot analysis and *in situ* mRNA hybridisation (Pritchard-Jones, 1992; Pritchard-Jones *et al.*, 1990). This gene, as described in chapter 1, is strongly expressed in the differentiating stem cells of the kidney, in the mesonephros, mesothelium, spleen and developing gonads, all tissues that undergo a mesenchyme-to-epithelium transition during their development. This pattern, together with the association of WT with abnormalities of the urogenital system, suggests that *WT1* mutations might have pleiotropic effects on both renal and genital development (van Heyningen *et al.*, 1990).

There are, however, several problems intrinsic to using human embryonic material: it is hard to obtain tissues from early and late gestational stages, to get enough samples of tissue of a particular age to be representative and to ensure that such material as can be collected is sufficiently fresh for there to have been no significant post-mortem changes. Moreover, for obvious ethical and practical reasons, it is impossible to use human material experimentally. These difficulties, which have limited the study of the role of *WT1* in development, are all surmountable with mouse embryonic material as fresh tissues can be obtained at any stage and, in addition, some mouse organ rudiments such as the kidney will, from their earliest stage, develop well in culture (for review, see Saxen, 1987). Furthermore, such evidence as we have, points to much of early development being similar in the two species.

The recent isolation of the mouse homologue of *WT1* has allowed this system to be utilised, and northern analysis has shown that there are high levels of the gene in the developing kidney and the adult spleen and low levels in the adult heart, lung, gonad and thymus, with the general level of kidney expression peaking at birth (Buckler *et al.*, 1991). A limited *in situ* mRNA hybridisation study has investigated the expression of *WT1* in the mouse genital system and found positive labelling in the non-germ cell components of both

the testis and the ovary and in the uterine wall or myometrium (Pelletier *et al.*, 1991a). This study also mentions briefly that the mesonephros and the mesothelial lining of the coelomic cavity express the gene.

In the hope that a detailed investigation of *WT1* expression would illuminate its function more clearly, a thorough *in situ* mRNA hybridisation study of the developing mouse has been undertaken to identify the temporal and spatial distribution of the gene from its earliest transcription and the results are presented in this chapter.

## 3.2 Results

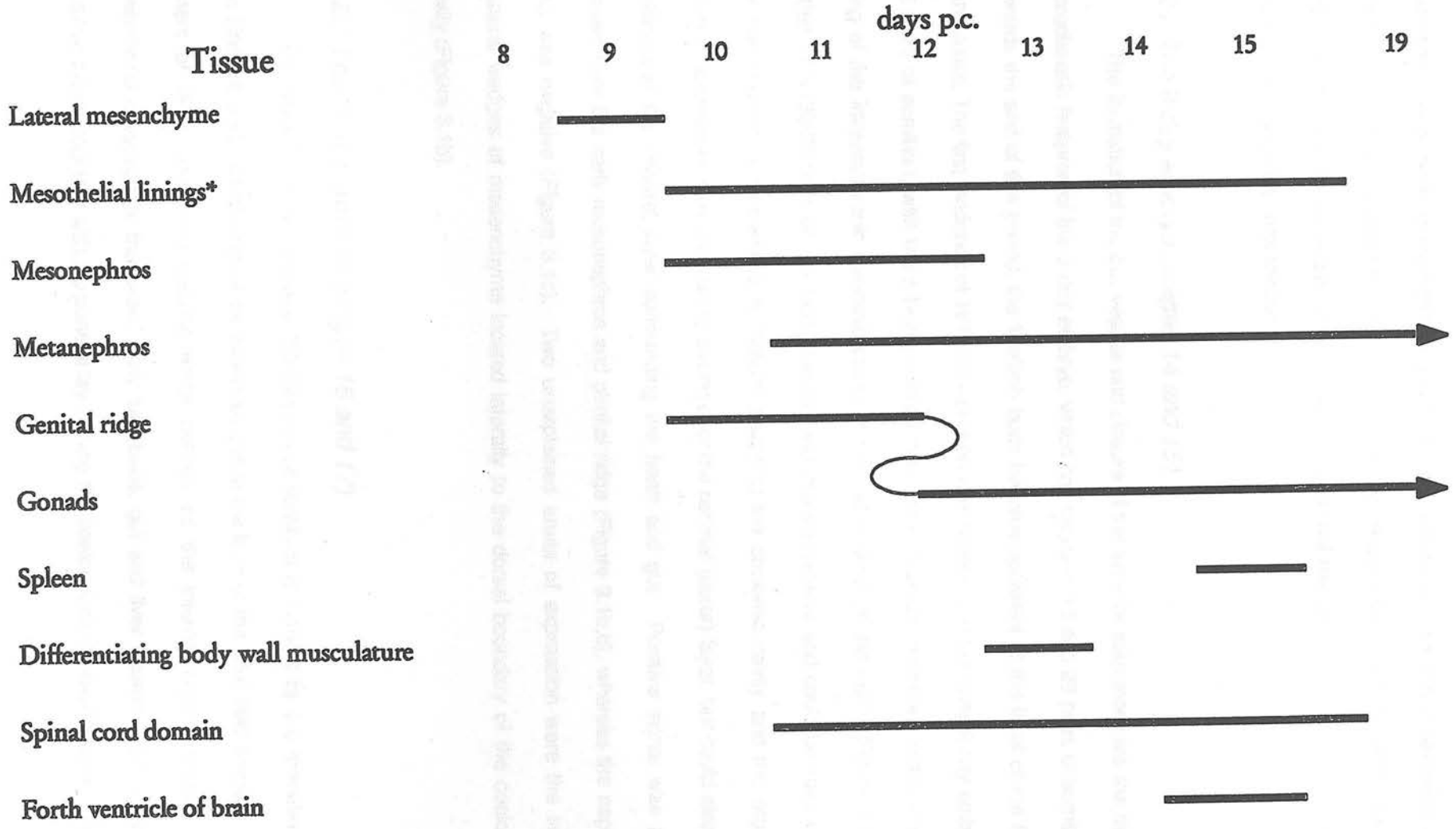
The data presented here detail the sites of *WT1* expression during mouse postimplantation embryogenesis from day 8 up until just before birth on day 19. The developmental stages (according to Theiler, 1989) are given in brackets and the tissues were identified using Kaufman's plates and Atlas (1990; 1992). Table 3.1 summarises the tissues where *WT1* was present as assayed by *in situ* mRNA hybridisation, using the anti-sense riboprobe described in chapter 2. The sense probe was used routinely as a negative control, with no specific signal being observed in any of the sections examined (e.g. Figure 3.4a). The metanephric kidney is mentioned briefly, but will be discussed in greater detail in chapter 4 in conjunction with the *in vitro* results.

### *3.2.1 The 8 day embryo (Theiler stages 12 and 13)*

This stage of development is distinguished by the initiation of somitogenesis (1-12 pairs of somites) and by neural-fold elevation, apposition and fusion (i.e. neurulation) which begins in the occipital and cervical regions. Transverse serial sections of 12 embryos were examined in 4 separate experiments, but no expression of *WT1* was seen in either the embryo itself, or in the extraembryonic membranes. The intermediate and lateral plate

Table 3.1

# WT1 Expression in the Developing Mouse Embryo




*includes heart, lung, liver, gut, mesorchium, pancreas etc

mesoderm, that give rise to the coelomic cavity and the tissues within it and in particular the urogenital system, are present in the 8 day embryo. As these tissues express *WT1* later in development, the negative results at this stage suggest that the gene is turned on at some time after this mesoderm has formed.

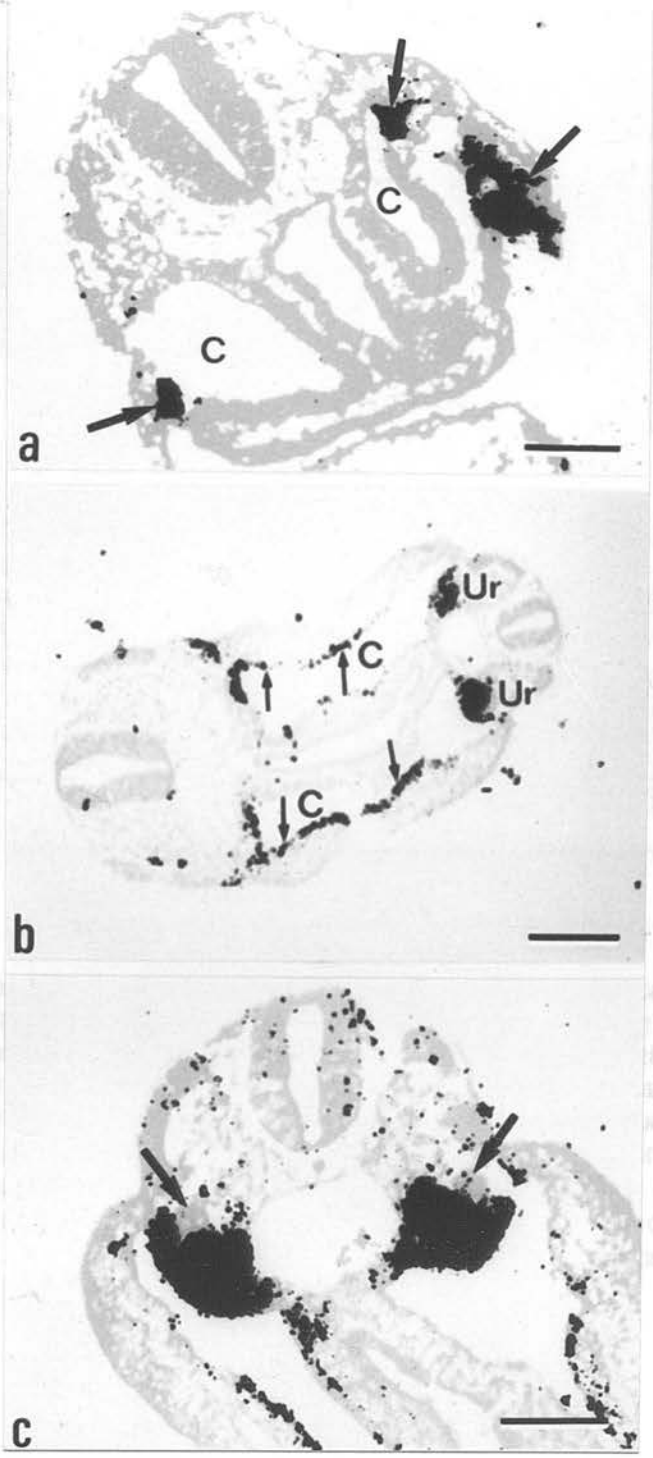
### *3.2.2 The 9 day embryo (stages 14 and 15)*

The formation of the otic vesicle and closure of the anterior neuropore are the most characteristic features of the 9 day embryo, which has between 13 and 29 pairs of somites. Towards the end of this period, the forelimb buds become apparent at the level of the 8th-12th somite. The first evidence of *WT1* transcription was observed in the early 9 day embryo (13 pairs of somites), with there being a small patch of expression restricted to the lateral lining of the intraembryonic coelomic cavity at the same level as the heart (Figure 3.1a). Within 12 h (20-25 pairs of somites), labelling was more extensive and could be seen over the mesothelium, a specialised epithelium bounding the coelomic cavity and the organs within it. Expression was particularly strong over the parietal (outer) layer, but could also be observed in the visceral layer surrounding the heart and gut. Positive signal was also present over the early mesonephros and genital ridge (Figure 3.1b,c), whereas the nephric duct was negative (Figure 3.1c). Two unexplained areas of expression were the small bilateral wedges of mesenchyme located laterally to the dorsal boundary of the coelomic cavity (Figure 3.1b).

### *3.2.3 The 10 day embryo (stages 16 and 17)*

This stage of embryogenesis (30-39 pairs of somites) is marked by the formation of the hindlimb buds, which appear as distinct bulges at the level of the 23rd-28th somite. The extent of *WT1* expression became better defined as the internal organs formed: the mesothelial coverings of the heart, early lung-buds, gut and liver showed high levels of positive signal, together with the parietal layer lining the coelomic cavity itself (Figure 3.2a,b).

Figure 3.1. Early expression of *WT1* in the developing mouse embryo. *In situ* mRNA hybridisation to transverse sections using the computer enhanced technique, with labelled regions shown in black. a) *WT1* was first expressed in the mesenchyme (arrows) lateral to the coelomic cavity (C) of an early 9 day embryo (bar: 100 μm). b) In the late 9 day embryo, the lining of the coelomic cavity showed positive labelling, with maximal expression at the lateral edge (arrows). *WT1* was also expressed in the urogenital ridge (Ur) that includes the early mesonephros (bar: 200 μm). c) High power micrograph of (b) showing that only the early mesonephric tubule and genital ridge were labelled, with the nephric duct (arrows) being negative (bar: 100 μm).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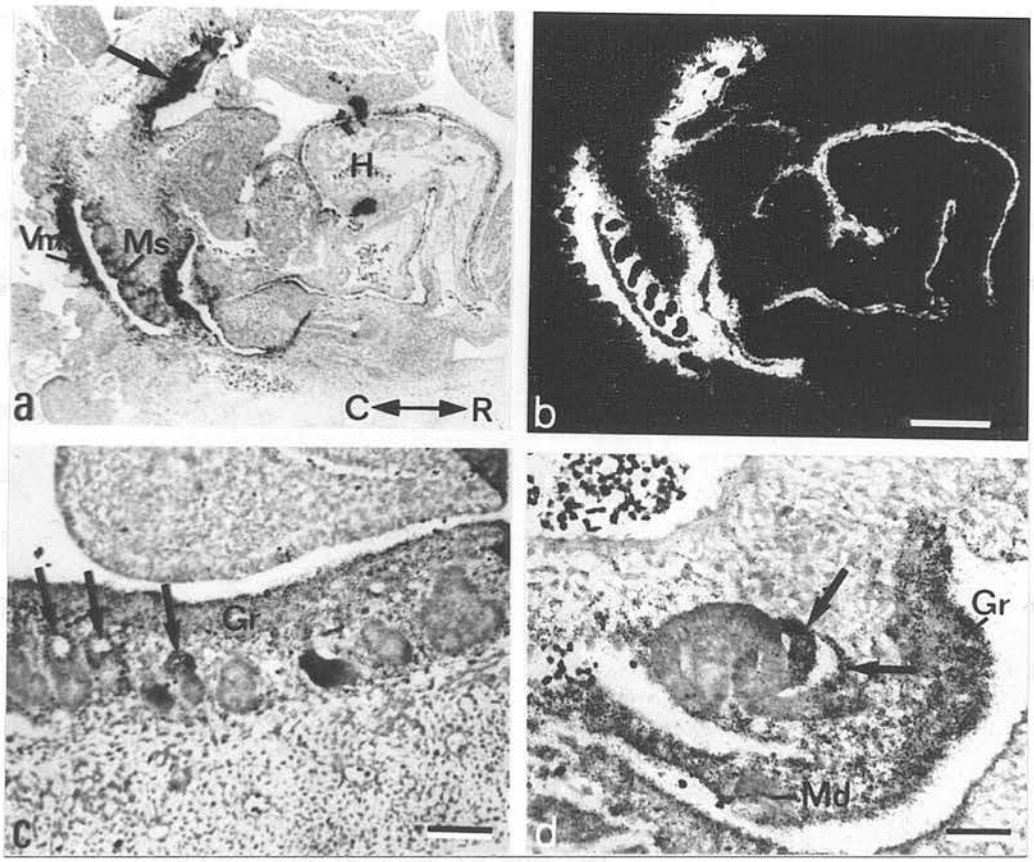


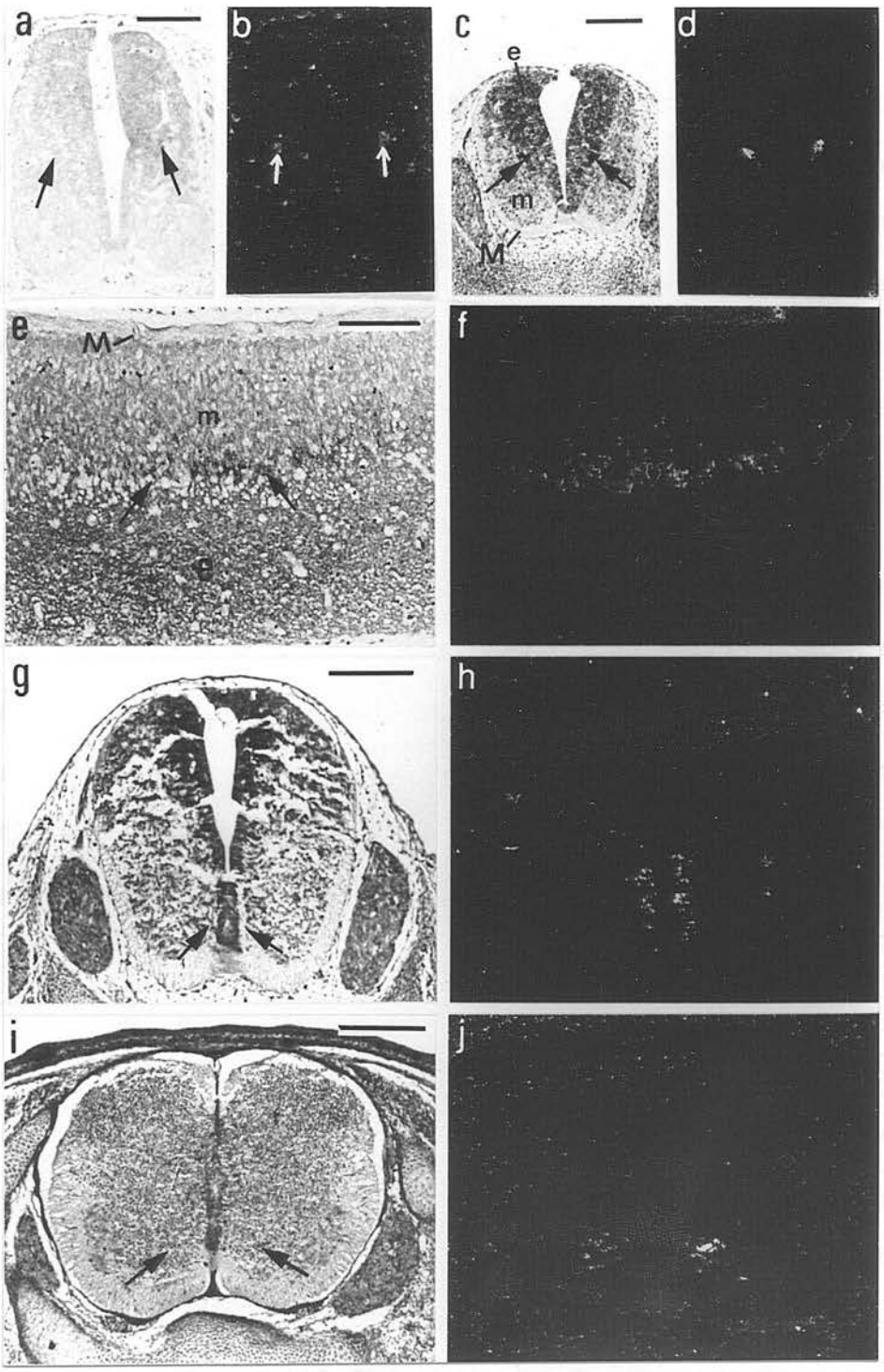

Figure 3.2. Expression of *WT1* in the mesonephros. a) Parasagittal section of a 10.5 day embryo (b, corresponding dark-field that reveals silver grains, marking the binding sites of the anti-sense probe). The mesonephros (Ms), the mesothelial coverings of the intracoelomic organs including the heart (H), the mesenchyme ventral to the spinal cord (Vm) and the cloaca (arrow) all expressed the gene. The rostral-to-caudal orientation (R,C) is given (bar: 400 μm). c) High power micrograph of an adjacent section to (a). The genital ridge (Gr), mesonephric mesenchyme and the glomerular pole of the mesonephric tubule (arrows) expressed the gene (bar: 100 μm). d) A transverse section through a mesonephric tubule illustrating the restriction of *WT1* label to the glomerular pole (arrows). The mesonephric duct (Md) did not express the gene (bar: 50 μm).

An area of undifferentiated mesenchyme ventral to the spinal cord also expressed the gene (Figure 3.2a,b) and positive signal was seen overlying mesenchyme that may represent the point where the mesonephric duct would later join the cloaca (arrow; Figure 3.2a,b). There was heavy labelling over the mesonephros (Figure 3.2a,b), with the tubules showing the characteristic pattern of expression previously described for the human embryo (Pritchard-Jones, 1992). The mesonephric tubules, particularly the rostral ones, are well-formed at this stage (Smith & Mackay, 1991) and, while the majority of the tubule did not express the gene, the pole associated with the glomerulus was heavily labelled (Figure 3.2c,d). The genital ridge at this stage was observed as a thickening of the coelomic epithelium adjacent to the mesonephros and this region expressed the gene at a high level (Figure 3.2c,d), with the mesonephric duct being unlabelled (Figure 3.2d). In a late example of an embryo of this stage, a low level of expression was observed in the metanephros. It is worth noting that *WT1* expression has been observed in the mesothelium of a 28 day human embryo (Pritchard-Jones, 1992), which corresponds to day 10 of mouse development.

### *3.2.4 The 11 day embryo (stages 18 and 19)*

During the 11th day of gestation (40->45 pairs of somites), the lens vesicle closes and becomes detached from the surface ectoderm and can be used as a criterion for recognising this stage of development. In the 11 day embryo, the pattern of *WT1* expression was maintained in all the tissues described for the previous day, with there being enhanced expression in the genital ridge and in the metanephric mesenchyme. In transverse sections, weak expression was apparent in a narrow linear domain of cells, running along the central region of the mouse spinal cord, at the boundary of the mantle and ependymal layers (Figure 3.3a,b). The longitudinal extent of this domain was, however, difficult to define, because the level of labelling was so low.

Figure 3.3. Expression of *WT1* in the spinal cord (b,d,f,h and j, are the corresponding dark-fields). a) Transverse section of a 11 day embryo, with patches of expression in the central region of the spinal cord (bar: 150 μm). c) Transverse section of a 12 day. Expression was in the same domain, which was at the border of the ependymal (e) and mantle (m) layer. The outer marginal layer (M) was unlabelled (bar: 200 μm). e) Sagittal section of an embryo from the same litter as (c), demonstrating the extent of the domain of expression (bar: 150 μm). g) Transverse section of a 13 day embryo. The domain of expression had moved ventrally towards the floor plate (bar: 300 μm). i) Transverse section of a 15 day embryo. Here, expression was observed in the ventral horn region of the spinal cord (bar: 300 μm).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### *3.2.5 The 12 day embryo (stage 20)*

This stage of development can be identified by the early stage of differentiation of the distal part of the limbs. In the forelimb, the handplate is seen to have an angular contour and the digital interzones demarcate the location of the fingers. In older embryos (stage 21) these interzones are more marked, and indentations are present between the tips of the fingers. At all stages, the footplate is markedly less advanced than the handplate, so that by stage 21, the footplate achieves the same level of differentiation obtained by the handplate at stage 20. The pattern of *WT1* expression in mice of this age was much as in the 11 day embryos studied. The mesonephros, which continued to express the gene, was regressing at the rostral end, with the most caudal tubules being highly convoluted. The genital ridge, which thickens considerably as the gonads form, showed very strong expression (Figure 3.4b,c) an observation confirming that of Pelletier *et al.* (1991a). Careful examination of the sections, revealed a slight variation in the level of signal overlying the gonads at this stage and may correspond to the morphological distinction of sexual differentiation that is first possible at this point in development. In contrast, both sets of genital ducts (mesonephric and paramesonephric ducts) were clearly negative (Figure 3.4b,c). Expression of the gene was again apparent in the small domain within the spinal cord, but the signal was more pronounced than it had been a day earlier (Figure 3.3c,d) and was uniform along the length of the cord (Figure 3.3e,f).

### *3.2.6 The 13 day embryo (stage 21)*

A characteristic feature of the 13 day embryo is the pronounced degree of indentation of the handplate. As before, there was strong expression of *WT1* over the mesothelial coverings of the major organs, with there being no suggestion of any decline in the level of signal. In the genital ridges, sexual differentiation was evident in the forming gonads: in both sexes, *WT1* expression was restricted to the developing sex cords and the overlying mesothelium (Figure 3.4d-f). No evidence of positive signal was observed in either

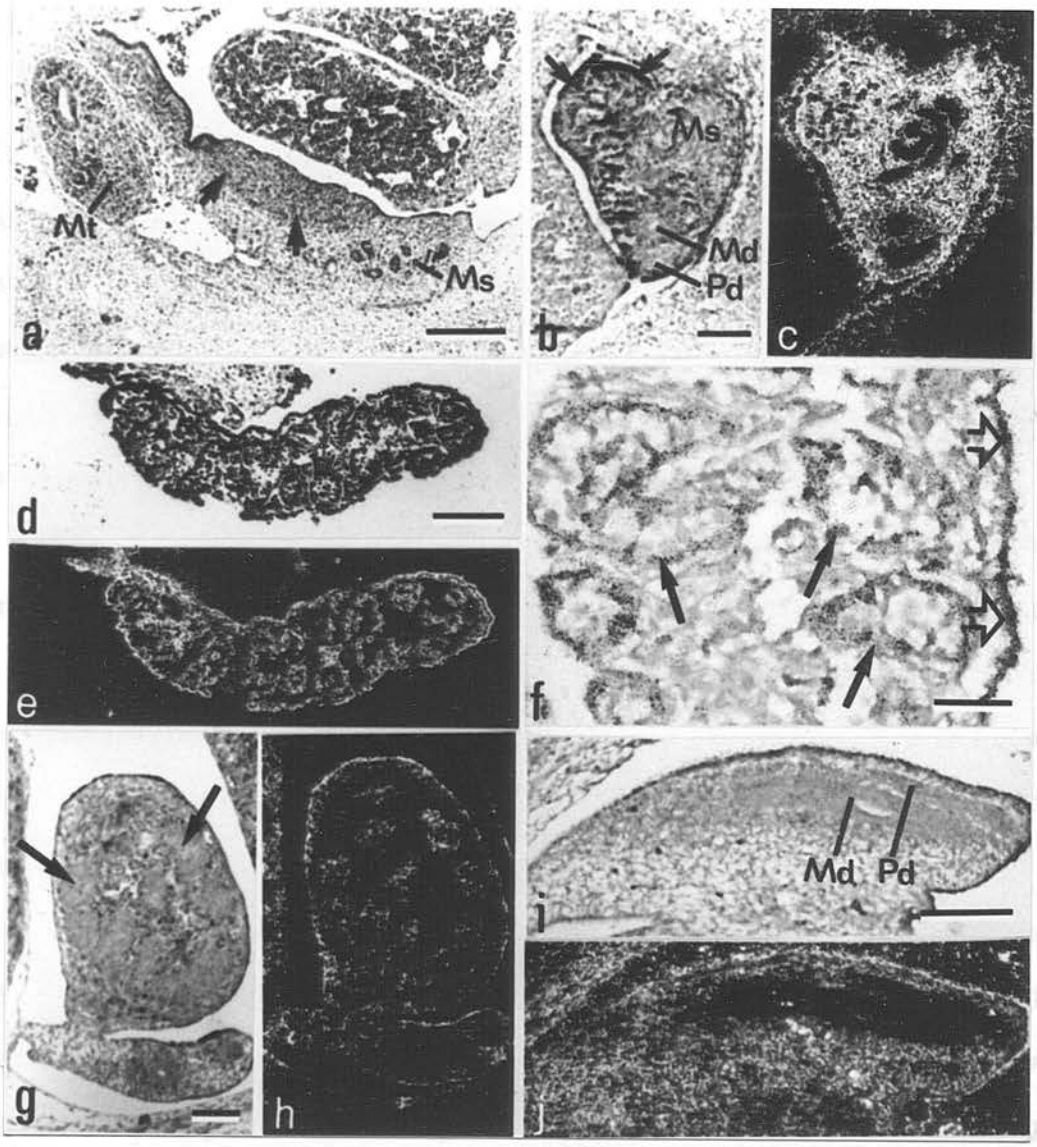



Figure 3.4. Expression of *WT1* in the gonads and genital ducts. a) Low power micrograph of a sagittal section of the 12 day genital ridge (arrows) hybridised with the sense probe, that was used routinely as the negative control and showed no specific labelling. The mesonephros (Ms) and metanephros (Mt) are also indicated (bar: 250 μm). b) High power of a transverse section through an embryo from the same litter as (a) demonstrating that the genital ridge (arrows) and mesonephros (Ms) were labelled, but that the genital ducts (Md, Pd) were not (c, dark-field; bar: 100 μm). d) By day 13 of development, *WT1* expression had become restricted to the epithelial component of the gonad (e, dark-field; bar: 200 μm). f) Same section at higher power, showing that the expression seemed to be limited to the sex cords (arrows) and overlying mesothelium (hollow arrows; bar: 50 μm). g) In the 15 day testis, positive signal was evident in the seminiferous cords (arrows) and the mesothelium (bar: 100 μm). i) The mesonephric (Md) and paramesonephric (Pd) ducts did not express the gene at any stage of their development, as illustrated by a 13 day embryo (bar: 150 μm).

the intervening loose mesenchyme (Figure 3.4d-f) or the genital ducts (Figure 3.4i,j). It was not possible in this study to determine unambiguously whether the germ cells expressed *WT1,* but this has been examined (Pritchard-Jones, 1992) in the $W^e$ mouse mutant that lacks germ cells, but has otherwise normal gonads. In male $W^e$ homozygotes, *WT1* expression was observed to be identical to that of AMH, a marker for Sertoli cells (Munsterberg & Lovell-Badge, 1991). Although this does not exclude *WT1* expression by the germ-cell component of the seminiferous tubules, it does indicate that any expression is at a relatively low level.

One area where expression was present for the first time was a region of condensed mesoderm which represented cells that were differentiating into the striated musculature of the body-wall (Figure 3.5). *WT1* transcription was transient in this domain, because 12 h later positive signal was no longer observed in the mesoderm of the body-wall, but was instead present in the zones that would later form the inter-thoracic muscles and furthermore, both areas were negative by day 15 of development.

Expression in the spinal cord extended along its length, but was more diffuse than it had been a day earlier and was clearly within the ventral region of the mantle layer. At this time, the morphology of the spinal cord was changing: the ependymal layer had narrowed and, in the ventral part, the mantle layer had expanded to form the ventral horn region (Figure 3.4g,h). A similar pattern has been observed in the spinal cord of an human embryo corresponding to this stage of mouse development (49 days; Pritchard-Jones, 1992).

### *3.2.7 The 15 day embryo (stage 23)*

The embryo at this stage can be distinguished by the digits of the limbs, that are clearly divergent and will not become parallel until day 17. The mesothelial coverings of the organs in both the pericardial and peritoneal cavities continued to express *WT1* (Figure 3.6). Although the spleen primordium can be identified by day 13 of mouse development, it was

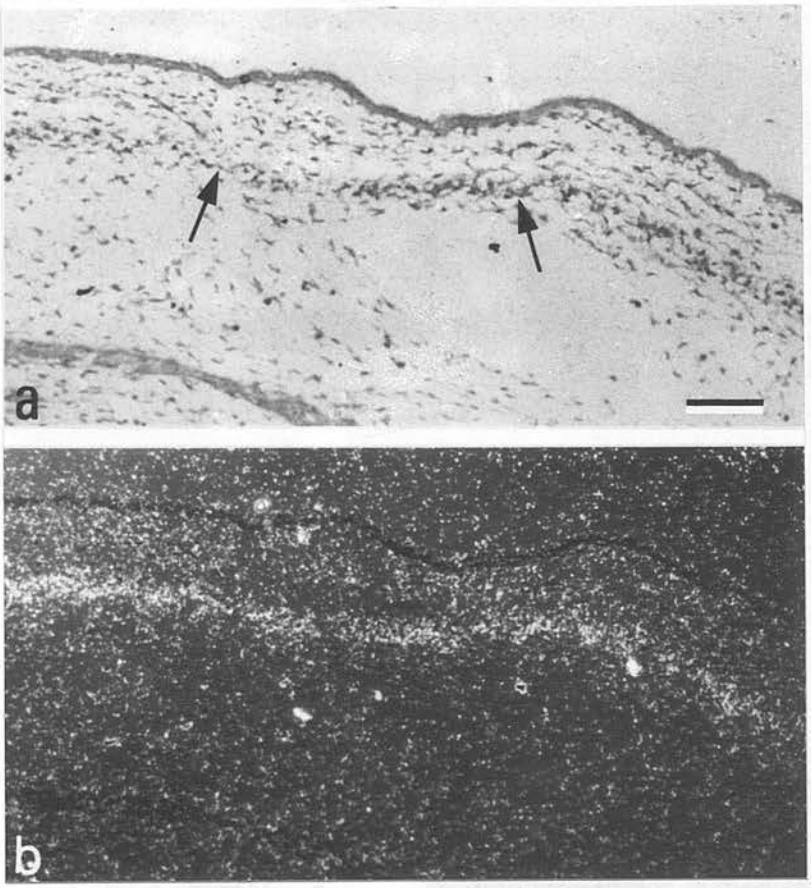



Figure 3.5. Expression of *WT1* in the developing musculature of the body wall. a) A line of cells (arrows) underlying the ectoderm showed positive labelling (b, dark-field; bar: 100 μm).

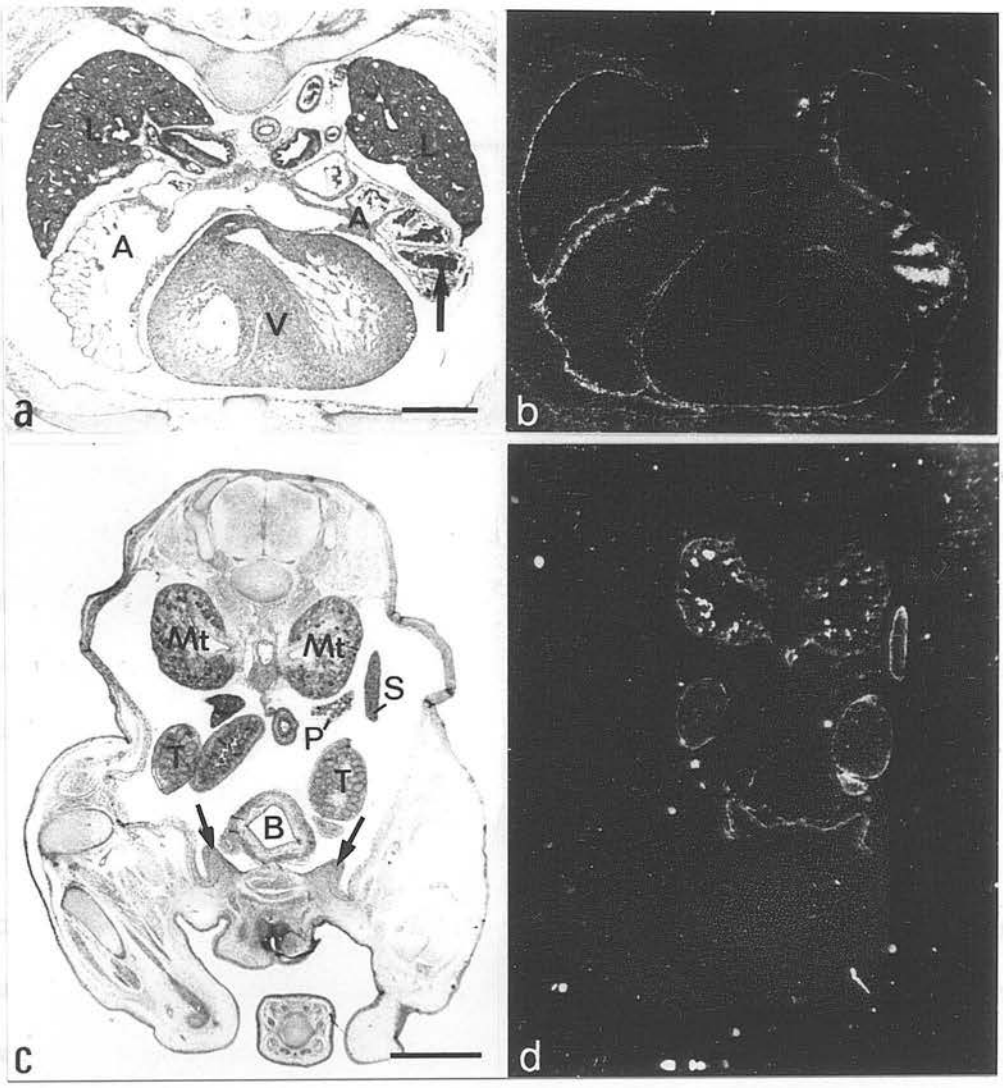



Figure 3.6. Expression of *WT1* in the 15 day embryo (b&d, dark-fields). a) Transverse section through the pericardial cavity showing that the mesothelial coverings of the lungs (L) and atria (A) and ventricles (V) of the heart were positively labelled. Red blood cells within the heart reflect light and are not actually labelled (arrow; bar: 500 μm). c) Transverse section through the lower peritoneum. The mesothelial coverings of the spleen (S), pancreas (P), bladder (B) and mesorchium (arrows) expressed *WT1* as did the metanephroi (Mt) and the testes (T; bar: 1 mm).

first distinguished in this study in the 15 day embryo. The mesothelium of the spleen expressed a high level of *WT1,* but no signal above the background level was observed in the rest of the organ (Figure 3.6c,d). Another tissue that was first identified at this stage of development and that expressed *WT1* was the mesorchium, the mesothelial-derived mesentery from which the testis was suspended (Figure 3.6c,d).

The domain of strongly expressing cells in the spinal cord was by this stage clearly located within the ventral horn region (Figure 3.i,j), and still extended caudally from the rostral extremity of the spinal cord to a region distal to the hindlimbs. Expression was particularly pronounced in the region of the hindlimbs, but no increase in the level of signal was detected in the corresponding area for the forelimbs. In addition, a small area of expression was observed in the brain of the embryo (Figure 3.7) and this was restricted to the diverticulum of the ependymal layer of the 4th ventricle, close to the rostral section of the medulla oblongata.

Perhaps the most surprising observation made in embryos of this age, however, was that the only three organs where gene expression had been turned off were the mesonephros, that had almost completely regressed, the non-glomerular components of the metanephric nephrons, that originated from the strongly expressing renal vesicles, and the condensed domain of body-wall mesoderm that had differentiated into muscle.

### *3.2.8 The 19 day embryo (stage 27)*

The length of gestation for the mouse is approximately 19 days and, in the light of the work showing that the level of *WT1* in the kidney reaches a peak at birth (Buckler *et al.,* 1991), an embryo of this age was examined. Here, *WT1* expression was substantially reduced with clear evidence of transcription being limited to the kidney where it continues into adulthood (the gonads and spinal cord were not examined). It was difficult to

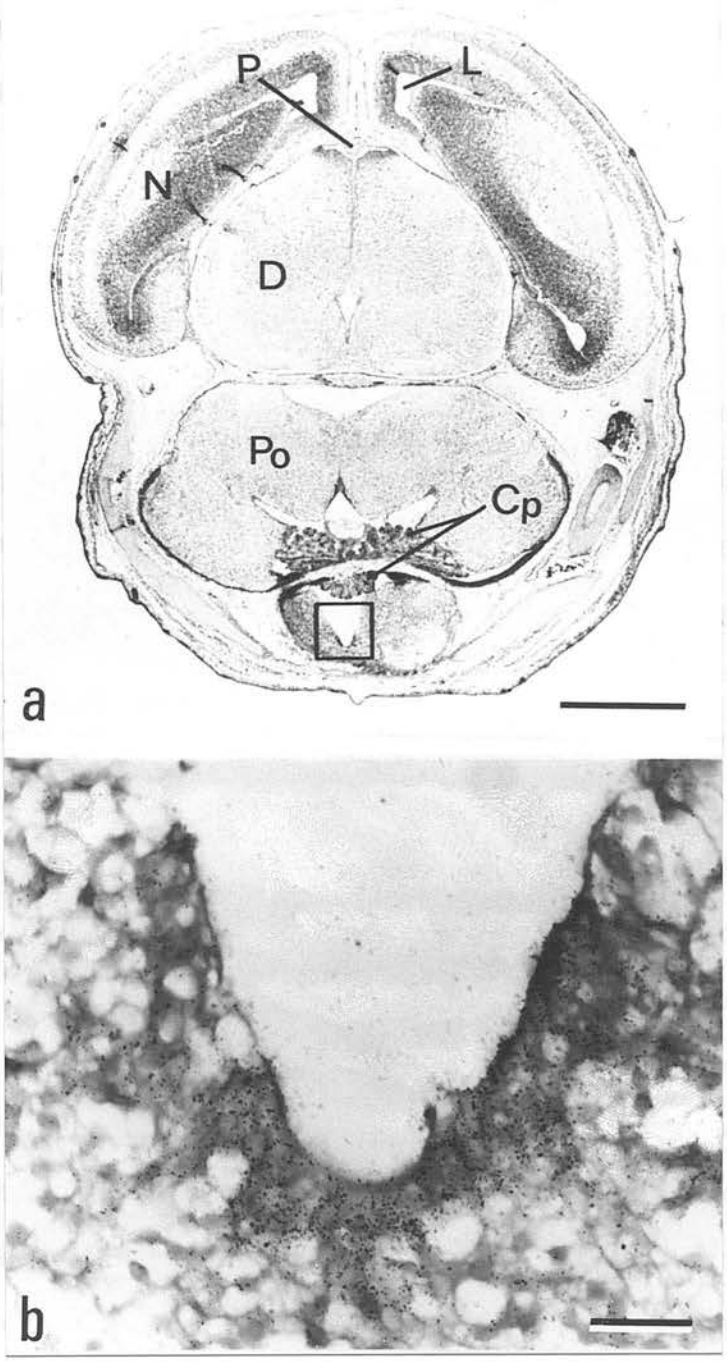



Figure 3.7. Transverse section through the brain of a 15 day embryo at the level of the 4th ventricle. a) A low power micrograph of the whole section. Note that the orientation is such that the top of the picture is ventral. Abbreviations; Cp, choroid plexus associated with roof of 4th ventricle, D, diencephalon (thalamus), L, lateral ventricle, N, neopallial cortex (future cerebral cortex), P, pineal recess of 3rd ventricle and Po, basal plate of pons (bar: 1 mm). b) Same section at higher power showing that only the roof of the 4th ventricle expressed *WT1* (bar: 50 μm).

determine whether the mesothelial coverings of the internal organs still expressed the gene, but if they did it was at a very low level.

## 3.3 Conclusions

In this chapter a systematic study of the expression of the WT predisposition gene, *WT1*, in the developing mouse using *in situ* mRNA hybridisation has been reported. It confirms published observations on the human fetus (Pritchard-Jones *et al.*, 1990), with the gene being expressed in essentially an identical pattern in the developing renal system, gonads and the mesothelial tissues of the coelomic cavity of the two species. For the mouse, however, it has been possible to examine much earlier tissue than has hitherto been feasible for the human embryo. Here, the temporal and spatial extent of *WT1* will be considered, with the implications of this work being discussed in the final chapter.

*WT1* transcripts were observed in two distinct sets of tissues that do not seem to be related in any obvious way. The first, as has previously been reported (Pritchard-Jones *et al.*, 1990; Pelletier *et al.*, 1991a), includes those associated with the mesenchyme-to-epithelium transformation within the body cavity, such as metanephric mesenchyme which becomes nephrogenic epithelium, the mesothelial cells that line the peritoneal, pleural and pericardial cavities and tissues (i.e. the lung, heart, liver, gut, spleen and other abdominal organs) and the epithelial cells of the gonads which are of mesodermal origin. The second set is a disparate collection of tissues that do not undergo such a transformation and include a line of cells within the spinal cord, a small domain of the 4th ventricle of the brain and the cells that will go on to form the striated musculature of the body-wall.

A feature common to most tissues expressing *WT1,* is that they are a subset of the mesoderm. Again, the rule is not universal because the gene is clearly expressed within the brain and in a domain of the spinal cord, both of which derive from ectoderm. The

expression of *WT1* in the spinal cord was first seen in the 11 day embryo, which is when the lateral motor columns are being formed, and this location is marked by alkaline phosphatase (AP) activity (Kwong & Tam, 1984). It is interesting that the domain of *WT1* expression lies inside the region of enzyme activity at this time and that the ventral movement of the *WT1* expression pattern over the period 13-15 days is roughly mirrored by the pattern of AP activity. The significance of these results cannot, however, be understood until the fate of these two domains is known.

Given, nevertheless, that most tissues where *WT1* transcripts are present are examples of mesenchymal differentiation, there are two other noteworthy features about its expression. The first is that the gene is turned on after early organogenesis has started and the second, is that it may never be fully turned off, even after the organs seem to have formed. *WT1* expression is first seen at about 9 day of mouse development in lateral boundary of the coelomic cavity. At this time, lateral mesoderm is well-established, 13 or so pairs of somites can be seen, the heart is forming, a gut is developing and the coelomic cavity, with its epithelial lining, has been present for at least 24 h. That *WT1* is turned on only after these organs have been initiated, argues that its role is limited to the development of secondary features of the tissues (i.e. mesothelial differentiation). At the other end of the temporal spectrum, *WT1* transcripts are present well after the tissues seem to have formed and the mesothelium differentiated, as the 15 day data shows. Indeed, in new-born and adult gonads (Pelletier *et al.,* 1991a) and in the epithelial podocytes of the renal corpuscle, *WT1* is still expressed at levels sufficient for detection using the technique of *in situ* mRNA hybridisation.

If we compare the results obtained in the present study, with those reported previously for the mouse by Buckler and co-workers (1991), several discrepancies emerge. They reported weak *WT1* expression in the adult heart, lung and thymus using northern analysis: in contrast, this study has shown that expression was restricted to the embryonic pericardium and pleura, with no evidence of positive signal being found in the 15 day

thymus. In the first two cases their observations may be accounted for by the mesothelial expression, but there seems to be no explanation for the thymus results. In addition, although the expression patterns in the developing mouse and human are very similar, there does seem to be a difference between the two species in the level of *WT1* in the spleen. In a 70 day human fetus, a high level of positive signal was seen in the mesothelial covering of the spleen and a slightly reduced level observed in the parenchyma of the organ (Pritchard-Jones *et al.*, 1990). In the mouse, however, *WT1* expression was limited to the mesothelium.

In conclusion, the results presented here have confirmed the importance of *WT1* in tissues undergoing a mesenchyme-to-epithelium transition and have described its expression in several tissues that do not fit into this category thus raising more questions about its function during development. Further insight into the role played by this gene might be obtained from studying the expression of *WT1* in the *in vitro* kidney culture system and in the following chapter such a study is described.

# CHAPTER 4

# THE EXPRESSION OF *WT1* IN THE METANEPHRIC KIDNEY

## 4.1 Introduction

The previous chapter examined the expression pattern of the *WT1* gene in the developing mouse embryo, but the metanephric kidney was only mentioned briefly and will be considered in more detail here. *In situ* mRNA hybridisation studies have already been carried out for the human embryonic kidney (Pritchard-Jones *et al.*, 1990) and to a very limited extent for the mouse (Pelletier *et al.*, 1991a) and these show that the gene is expressed in both species in a specific pattern during nephrogenesis: it is produced at a low level in the undifferentiated stem cells of the outer cortex and at a higher level in the renal vesicle where it eventually becomes restricted to the podocyte cells of the visceral layer of the renal corpuscle.

One of the great advantages of the mouse is that its metanephric kidney will develop in culture to much the same extent that it would have done *in vivo*. The expression pattern of the *WT1* gene has yet to be examined in this system and the need for such a study is apparent when we consider the amount of experimental work that has been carried out using it and the wealth of knowledge generated (see chapter 1, for details). Moreover, the *in vitro* system lends itself to experimental manipulation and, as discussed previously, its use has facilitated the study of the mechanisms underlying nephrogenesis.

The results presented in this chapter couple a detailed study of the expression of *WT1* in the developing kidney of the mouse embryo itself (from day 11 until day 19) with that of the metanephric rudiment in culture using *in situ* mRNA hybridisation. In order to examine the involvement of *WT1* in induction, the key event underpinning organogenesis of the metanephros, the *in vitro* transfilter culture system was used. In addition, the pattern of *WT1* expression *in vitro* has been used to assay the effects of two molecules, LIF and cytochalasin B, that have previously been shown to disrupt nephrogenesis (Bard & Ross, 1991; Bard, 1990a). By studying *WT1* transcription in rudiments cultured under these experimental conditions, it has been possible to determine more clearly the point in development at which inhibition occurred.

## 4.2 Results

### *4.2.1 The pattern of* WT1 *expression* in vivo

The formation of the mouse metanephric kidney begins very early on day 11 of gestation when the ureteric bud invades the domain of condensed cells that constitutes the metanephric mesenchyme (refer to Figure 1.2 in chapter 1, for details). The results from the *in situ* mRNA hybridisation experiments reported in chapter 3 had indicated that, in embryos of this developmental stage when induction has probably not yet occurred (see section 4.2.2), there was a low level of *WT1* expression over the entire metanephric mesenchyme (Figure 4.1). In the 12 day kidney, where induction has taken place and the ureteric bud undergone several bifurcations, the cap of condensed mesenchyme surrounding the ampullae expressed the gene at a high level (Figure 4.2a,b). In some examples, the whole of the mesenchyme seemed to be labelled (Figure 4.2a,b), but in other cases only the cells immediately adjacent to the bud expressed the gene. An additional observation that held true throughout kidney development was the absence of positive signal over the ureteric bud and its branches.

By day 13 of development, the initial stages of nephron formation could be observed (for details, see Figure 1.2). The undifferentiated stem cells at the periphery of the kidney expressed *WT1* and a specific pattern was observed in the nephrogenic epithelium (Figure 4.2c). There was uniform positive signal over the whole renal vesicle and this then became localised to the cells of the presumptive Bowman's capsule as first the comma- and then the S-shaped body formed (Figure 4.3a-c). Careful examination of the tissue at this stage, showed that the level of positive signal was substantially higher in the differentiating nephron than in the stem cells of the outer cortex (Figure 4.2c). After a further 24 h, the basic histoarchitecture of the organ was evident (Figure 4.2d). The stem cells in the cortex of the 14 day kidney continued to express *WT1,* but at a level that was considerably lower than that observed in Bowman's capsule of the developing nephron (Figure 4.2d,e). It is

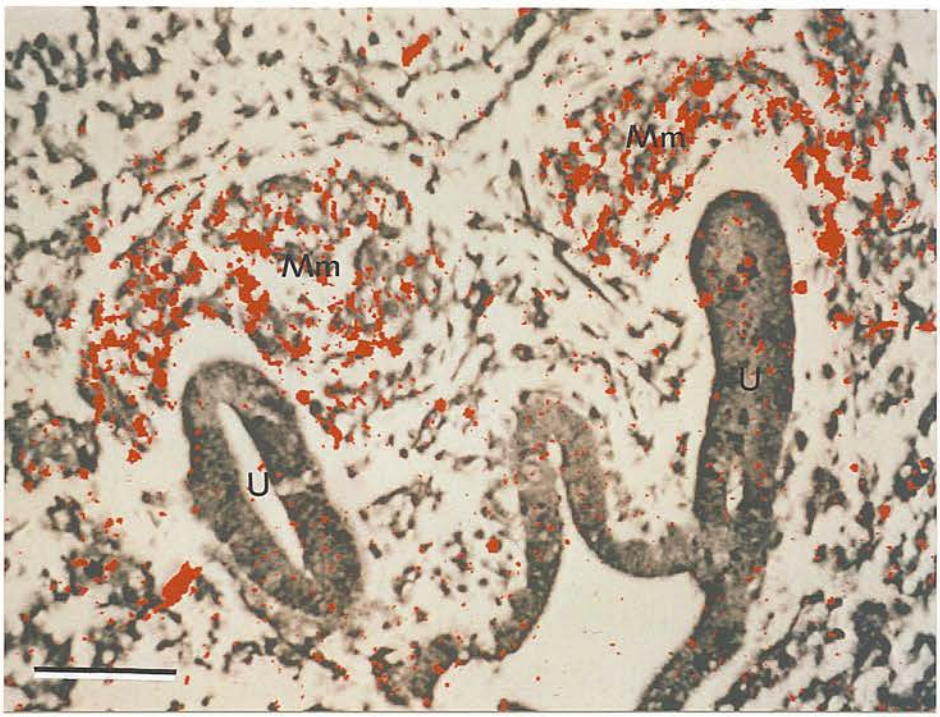
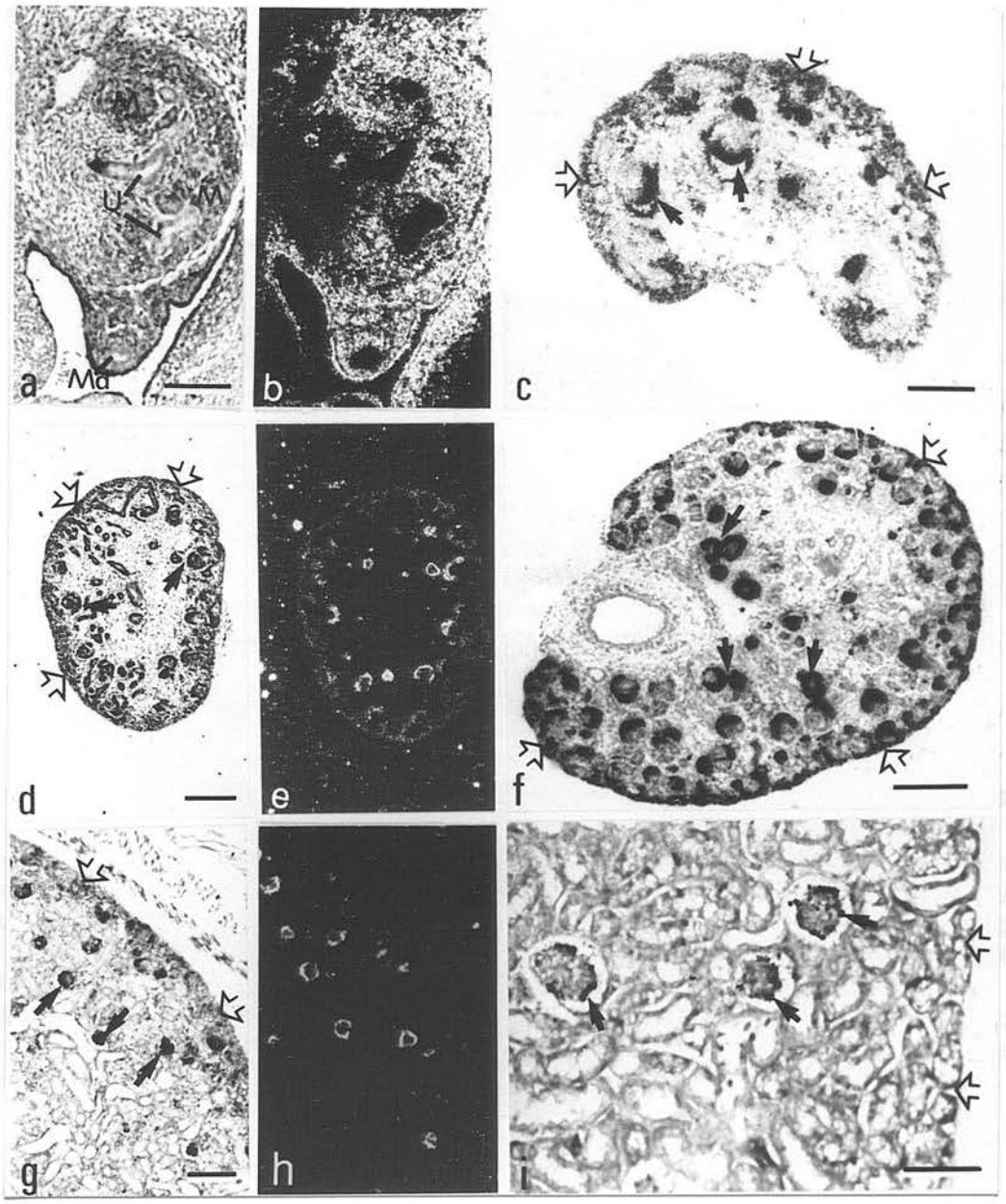



Figure 4.1. Expression pattern of *WT1* in the mouse metanephros before induction. *In situ* mRNA hybridisation to a transverse section of an early 11 day embryo using the computer-enhanced technique, with positive signal demonstrated in red. Both the right and left kidneys are shown, with each ureteric bud (U) surrounded by a cap of metanephric mesenchyme (Mm; bar: 100 μm).

Figure 4.2. Localisation of *WT1* mRNA in the developing metanephros. a) In the 12 day embryo, positive signal was restricted to the metanephric mesenchyme (M) that had segregated around the ureteric bud (U). The mesonephric duct (Md) did not express the gene (b, dark-field; bar: 150 μm). c) A day later, both the undifferentiated stem cells of the outer cortex (hollow arrows) and the developing nephrons (arrows) expressed *WT1* (bar: 100 μm). d) A section of a 14 day kidney, with labelling evident in both the cortex (hollow arrows) and Bowman's capsule of the forming nephron (arrows; e, corresponding dark-field; bar: 100 μm). f) The 16 day kidney showed a similar pattern of expression (bar: 200 μm). g) In the 19 day kidney, positive signal was only just visible in the cortex (hollow arrows), but was still clearly observed in the renal corpuscle (bar: 150 μm). i) A section of an adult kidney showing that *WT1* expression was only maintained in the podocytes (bar: 150 μm).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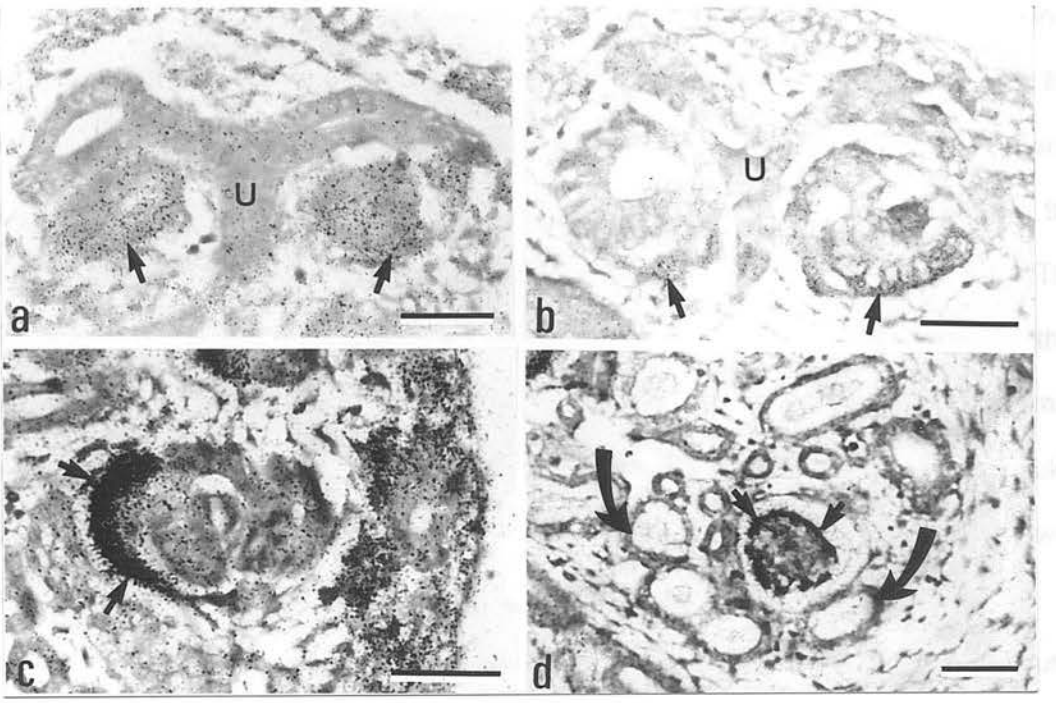



Figure 4.3. A series of sections from 13-16 day embryonic kidneys illustrating the expression pattern of *WT1* during nephrogenesis (all bars: 50 μm). a) Renal vesicles (arrows), that always form on the medullary side of the ureteric bud (U), were uniformly labelled. b) In the comma-shaped body (left), *WT1* expression was confined to the epithelium of the future Bowman's capsule (arrow). The aggregate on the right may have developed further to the S-shaped body and demonstrates that positive signal was still restricted to the cells of the presumptive Bowman's capsule (arrow). c) A S-shaped body that had elongated and convoluted, showing that the gene was localised by this stage to the visceral layer of Bowman's capsule (arrows). d) A nephron that seems to be fully mature (curved arrows), illustrating that *WT1* mRNA was confined to the podocyte cells (arrows).

important to note, that after the renal vesicle stage, labelling became restricted to the cells of the future renal corpuscle and was never observed in any other section of the nephron.

Kidneys from 15, 16 and 17 day embryos were also examined and in these the temporal and spatial gradient of nephron formation was clearly demonstrated. Within a single section, the complete spectrum of development could be observed; from the undifferentiated stem cells of the outer cortex, through the increasingly more complex renal aggregates to the seemingly mature nephrons in the medulla. In such material, the relationship between nephron formation and *WT1* expression was evident (Figure 4.2f). The stem cells continued to express the gene, although it was difficult to determine whether these cells still formed a continuous layer by this stage. The most central nephrons may have completed their development, because sections of the distal and proximal tubules could be seen lying adjacent to the renal corpuscle and in these cases the gene was restricted to the podocyte cells of the visceral layer of Bowman's capsule (Figure 4.3d).

In the 19 day kidney, the level of *WT1* mRNA was only just discernible in the outer cortex, although the podocytes of the renal corpuscle continued to express the gene (Figure 4.2g,h). The adult kidney was also examined, because northern blot analysis had shown that the gene was expressed at a low level in this tissue (Buckler *et al.*, 1991). The only evidence of positive signal found in the present study was restricted to the podocytes (Figure 4.2i).

If we consider the results from the *in vivo* study, several key observations emerge. First, it is clear that the pattern of *WT1* expression in the mouse is very similar to that described previously in the human (Pritchard-Jones *et al.*, 1990). The single ambiguity lies in the status of the uninduced metanephric mesenchyme, where observations based on human fetal tissue, whose fixation is often delayed, had led to the conclusion that the level of expression was not significantly above background (Pritchard-Jones *et al.*, 1990). The question of whether *WT1* is expressed before induction in the mouse is examined further in the next section. Second, the level of *WT1* mRNA increases significantly after induction and then becomes expressed at a higher level still in the forming nephron, indicating that this

gene is transcribed at three different levels during the development of the kidney. Third, as the nephron matures, *WT1* expression becomes restricted to the visceral epithelial layer of the renal corpuscle where it is maintained into adulthood. Finally, the fact that the gene is still transcribed at 19 days of development, even at a low level, in cells at the periphery of the kidney suggests that in the mouse, nephrogenesis continues into the neonatal period, albeit at a reduced rate.

### *4.2.2 The relationship between* WT1 *transcription and induction*

The results from the *in vivo* analysis imply, but do not prove, that *WT1* is present before the metanephric mesenchyme is induced. In order to clarify this situation, the expression pattern of this gene was studied in the transfilter system, where the effect of induction on the metanephric mesenchyme could be analysed directly.

This *in vitro* system of kidney culture has already been described in some detail (chapters 1 and 2), but it is worth considering the experimental design again briefly. Uninduced metanephric mesenchymes were isolated from early 11 day embryos and the ureteric buds removed. A proportion of the mesenchymes were fixed at this point, to enable the level of *WT1* expression in the initial uninduced tissue to be determined. The rest were then either cultured alone or with an heterologous inducer, in this case the embryonic spinal cord, attached to the opposite side of a porous filter. The length of time that the rudiments were cultured for was carefully determined to allow both the initial response to induction to be measured (24 h) and the long-term effects of the interaction between the mesenchyme and the inducer to be examined (96 h).

The results presented here describe the material from two independent transfilter experiments that were divided to enable 3 separate *in situ* mRNA hybridisation experiments to be carried out, with the results being summarised in Table 4.1. They show that *WT1* was expressed at a low level prior to induction (Figure 4.4a), but while two of the experiments were unequivocal, the third indicated that the gene was not expressed at this point in development. In all three experiments, the level of positive signal increased significantly

Table 4.1. Summary of the results from the transfilter experiments.

| | 1st experiment | 2nd experiment | 3rd experiment |
|---|---|---|---|
| 0 h | - | + | + |
| 24 h: no inducer | - | + | + |
| 24 h: inducer | + | ++ | ++ |
| 96 h: no inducer | - | - | - |
| 96 h: inducer | ++ | +++ | +++ |

| | |
|---|---|
| - | no expression above background |
| +, ++, +++ | level of positive signal |

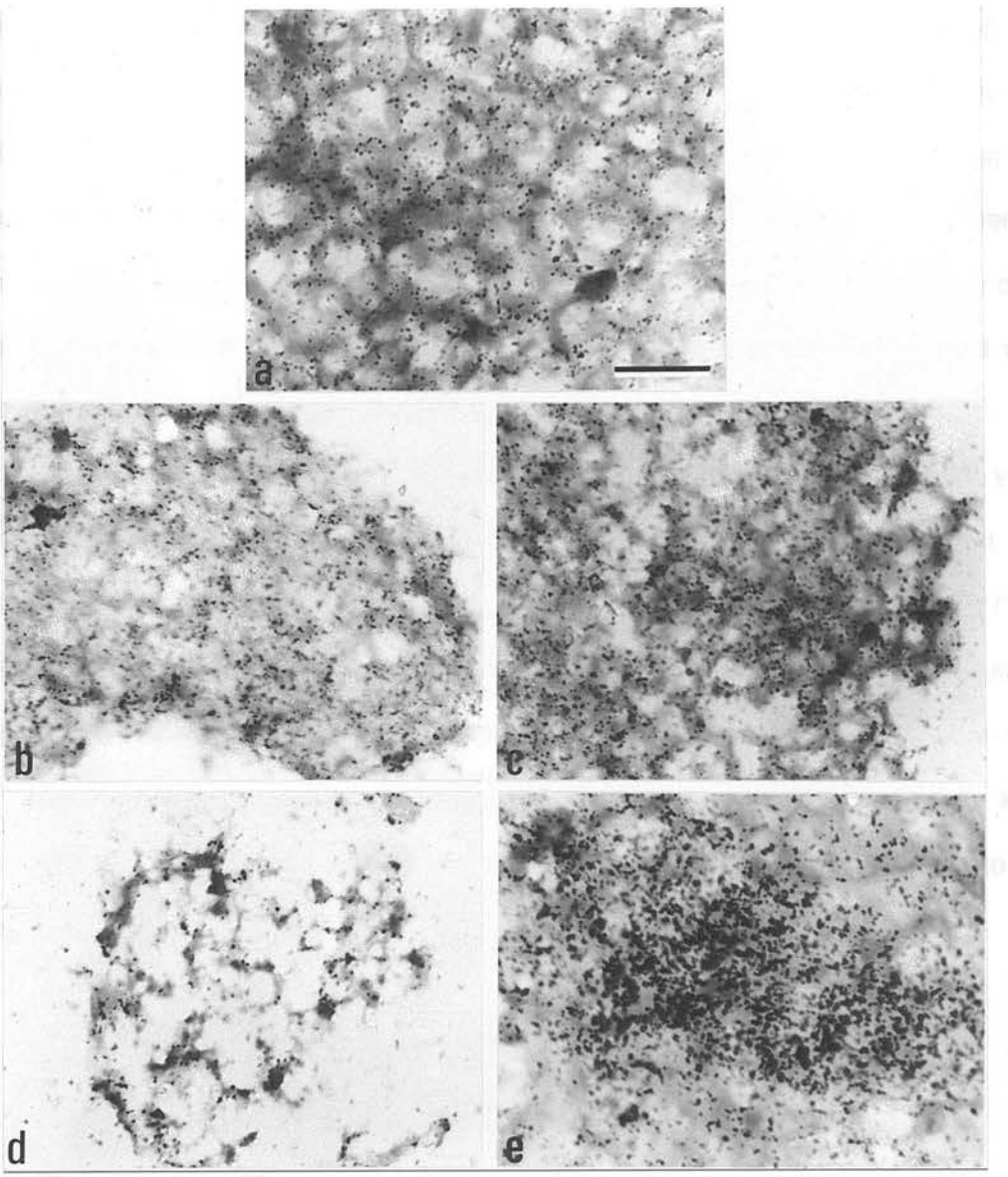



Figure 4.4. Expression of *WT1* in the *in vitro* transfilter culture system. Note that all the sections were photographed at the same magnification (bar: 30 μm). a) In the metanephric mesenchyme removed prior to induction, a low level of *WT1* expression was observed. b) In mesenchyme cultured for 24 h without an inducer, a low level of positive signal comparable to that shown in (a) was seen. c) After 24 h in culture with an inducer, the level of *WT1* expression had increased. d) Mesenchyme cultured without an inducer for 96 h, showed poor survival and little if any evidence of *WT1* expression. e) After 96 h in culture with an inducer, an high level of positive signal was observed, with certain areas showing a greater density of silver grains than others.

after exposure of the mesenchyme to the inducer for 24 h (Figure 4.4c). In contrast to the uniform labelling of the mesenchyme seen after 24 h of culture with an inducer, the experiments carried out over 96 h demonstrated an increase in *WT1* expression that was restricted to the areas of mesenchyme that had aggregated and were undergoing the transition to epithelial cells (Figure 4.4e). Finally, although the initial level of mRNA seemed to be maintained after 24 h in culture without an inducer (Figure 4.4b), this was not the case following 96 h under the same conditions (Figure 4.4d), where the tissue survived very poorly and the level of positive signal was negligible.

Two main points emerge from this data. First, in the absence of inducer, the initial low level of *WT1* transcription declines and the tissue degrades, proving that the mesenchyme was uninduced when it was isolated. It is therefore clear that *WT1* is expressed in uninduced mesenchyme, but its further production is contingent on induction taking place. Second, the data from the experiments where the mesenchyme was recombined with the inducer, shows that the effect of induction is to stimulate *WT1* transcription throughout the tissue, but that this production later becomes restricted to the forming nephrons.

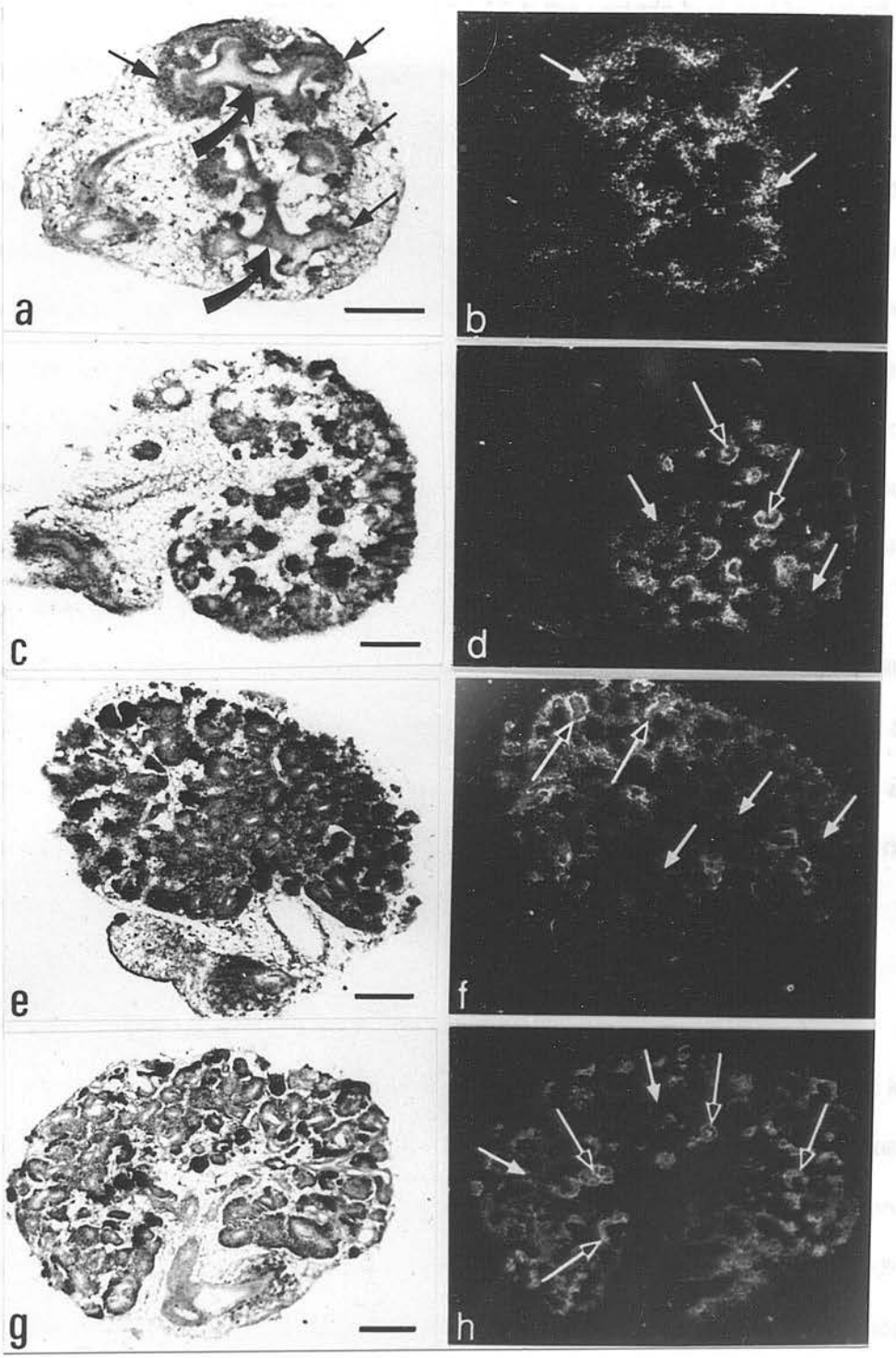
### *4.2.3 The expression of* WT1 *in the* in vitro *whole-organ system*

#### 4.2.3.1 Culture in routine medium

The pattern of *WT1* expression in metanephric rudiments cultured in routine medium was studied over the course of a 6 day period, with the tissue being examined daily. For these experiments, the kidneys were dissected from late 11 day embryos and in contrast to the transfilter experiments, the mesenchyme and ureteric bud components were left intact. The changes that occurred in routine culture were useful in two respects; they enabled the details of expression to be clarified and they served as a control for the experiments described in the following sections.

After 24 h in culture the ureteric bud had undergone several bifurcations, with each tip being surrounded by a cap of condensed mesenchyme (Figure 4.5a). In the material that

Figure 4.5. Expression of *WT1* in the whole-organ *in vitro* system (b,c,f,h, dark-fields). a) A section of a metanephric rudiment cultured for 24 h. The ureteric bud (curved arrows) had bifurcated several times, while the condensed mesenchyme surrounding each tip expressed *WT1* (arrows; bar: 150 μm). c) After 72 h, the ureteric bud had branched many times and was associated with extensive areas of condensed mesenchyme (arrows). Several aggregates undergoing the mesenchyme-to-epithelium transition (hollow arrows) could be seen and these expressed the gene at a higher level than the condensed mesenchyme (bar: 150 μm). e) In a rudiment cultured for 96 h, the pattern described in (c) was more striking (bar: 100 μm). g) By 144 h, in addition to areas of condensed mesenchyme (arrows), there was evidence that *WT1* had become restricted in the more mature nephrons to Bowman's capsule (hollow arrows; bar: 100 μm).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had been cultured for 72 h there was evidence of aggregates having formed that have been shown in previous studies (Ekblom *et al.*, 1980a; 1981a) to be undergoing the transition to nephrogenic epithelium (Figure 4.5c). This pattern became more pronounced in the rudiments cultured for longer periods, so that by 96 h the ureteric bud had branched many times and was associated with extensive areas of condensed mesenchyme (Figure 4.5e). In addition, a number of nephrons were observed and these have been demonstrated by this stage to have developed many of the features characteristic of this specialised epithelium (Ekblom *et al.*, 1980a; 1981a). The data from the *in situ* mRNA hybridisation experiments showed that the cap of condensed mesenchyme surrounding the ampullae of the collecting system expressed *WT1* at a level that increased substantially as the cells underwent the transformation to epithelium (Figure 4.5a-h). It was difficult to distinguish in all cases, because of the poor morphological detail preserved after the hybridisation treatment, but in a couple of rudiments cultured for 144 h, positive signal was seen to be restricted to the inner layer of Bowman's capsule (Figure 4.5g,h).

From these experiments it is clear that the expression of *WT1 in vitro* is the same as that observed *in vivo*. It is also worth noting that these rudiments are avascular and as the pattern of *WT1* expression in the renal corpuscle is not altered significantly from the *in vivo* results, confirms that this structure will develop to a great extent in the absence of endothelial cells (Bernstein *et al.*, 1981).

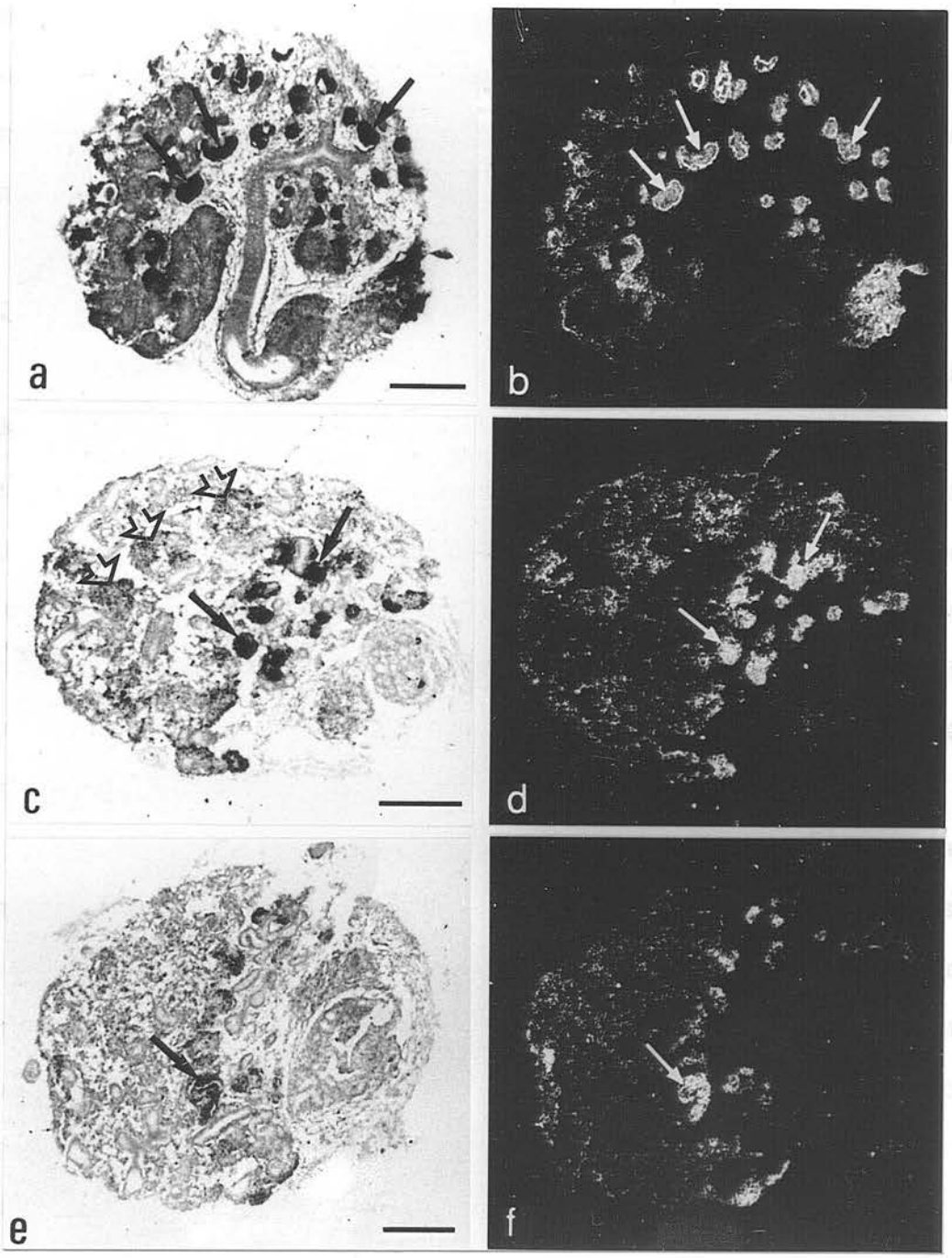
#### 4.2.3.2 The effect of LIF

One of the main advantages of studying the development of the metanephric kidney *in vitro*, is that experimental manipulation can be carried out in a controlled environment. It is relatively simple to add a defined substance to the culture medium and then observe the resulting changes. Here, such a study was carried out using LIF and the rudiments subsequently examined for their pattern of *WT1* expression. This served two purposes; it investigated the use of *WT1* as an assay for kidney development and in doing so enabled the effect of LIF on the formation of the nephron to be more clearly defined.

LIF, inhibits embryonic-stem (ES) cell differentiation in culture (Smith *et al.,* 1988; Williams *et al.,* 1988) and has been shown to affect the development of leukaemic, osteogenic and neuronal cells *in vitro* (Abe *et al.,* 1986; Moreau *et al.,* 1988; Yamamori *et al.,* 1989), suggesting that it may have a role in the control of a variety of pluripotent mammalian cells. In a recent study, metanephric rudiments cultured in LIF were examined and it was found that this glycoprotein had little effect on growth and may have stimulated the bifurcation process of the ureteric bud, leading to an increase in the number of collecting ducts (Bard & Ross, 1991). They did report a much more pronounced effect on the mesenchyme, however, with LIF used at 4 times the concentration needed to inhibit ES-cell differentiation (4xLIF) reversibly blocking the effect of induction on the mesenchyme. Although the mesenchyme condensed around the ampullae, the number of nephrons formed over a 6 day period was reduced by at least 75%. The fact that the few nephrons that did form were grouped around the base of the ureteric bud, led to the suggestion that these originated from mesenchyme that had already been induced at the time of dissection from the embryo. In addition, an unusually high number of aggregates that expressed laminin around their basal surface were found and indicate that LIF could block nephrogenesis at a second point.

In the present study, embryonic kidneys were cultured for 6 days in routine medium (this tissue acted as a control, see previous section for details), 4 x LIF and 8 x LIF. In rudiments cultured in 4 x LIF (Figure 4.6c,d), the condensed mesenchyme surrounding the collecting system expressed *WT1* at a level comparable to that observed in the control tissue (Figure 4.6a,b). There were also fewer epithelial aggregates expressing a high level of *WT1* and these tended to be grouped around the basal area of the ureteric bud (Figure 4.6c,d). Finally, there was some evidence of aggregates that expressed *WT1* at an intermediate level (Figure 4.6c,d). The results from the rudiments cultured in 8 x LIF were similar to those described for the 4 x LIF, but a more extreme effect on the expression pattern of *WT1* was observed, so that very little evidence of epithelialisation was seen (Figure 4.6e,f).

Figure 4.6. The effect of LIF on the expression pattern of *WT1 in vitro* (b,d,f, dark-fields; all bars: 200 μm). a) A control rudiment cultured in routine medium, shows that many nephrons had formed and these expressed a high level of *WT1* (arrows). c) A section of a rudiment cultured in 4 x LIF. In comparison to the control (a), there were fewer areas where a high level of positive signal was observed (arrows). An intermediate level of signal was seen in several areas of condensed cells (hollow arrows). e) There was little evidence of the mesenchyme-to-epithelium transition having occurred (arrow) in a rudiment cultured in 8 x LIF.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These results show that LIF initially blocks nephrogenesis at a point downstream of induction, because not only does the mesenchyme condense around the tips of the branching ureteric, but it also expresses *WT1* at a level that is indistinguishable from similar areas of the control tissue. The observation of aggregates that express the gene at an intermediate level, confirms the suggestion that LIF blocks this process at a second point (Bard & Ross, 1991) and demonstrates that this is almost certainly at a stage of nephron formation after the renal aggregate has undergone the transformation to epithelium.

#### 4.2.3.3 The effect of cytochalasin B

In addition to LIF, experiments were carried out in which rudiments were cultured in medium containing a low concentration of cytochalasin B (0.2 µg/ml), that is known to disrupt microfilament integrity and inhibit filament assembly. Used at such a low level, this drug was sufficient to weaken the cytoskeleton of the metanephric rudiment cells, but was not enough to affect either cell growth or division of the ureteric bud. In earlier experiments it had been reported that, although the mesenchyme in such rudiments formed the initial aggregates, these did not go on to become nephrogenic epithelium in the majority of cases (Bard, 1990a).

The results from the *in situ* mRNA hybridisation experiments undertaken here, demonstrated that in rudiments cultured in cytochalasin B the areas of condensed mesenchyme surrounding the tips of the branched collecting system expressed *WT1* at a comparable level to the control tissue, but that there were fewer epithelial aggregates (Figure 4.7). This suggests that cytochalasin B inhibits nephrogenesis at a point downstream of induction, but prior to the transition of the mesenchymal aggregates to epithelial cells.

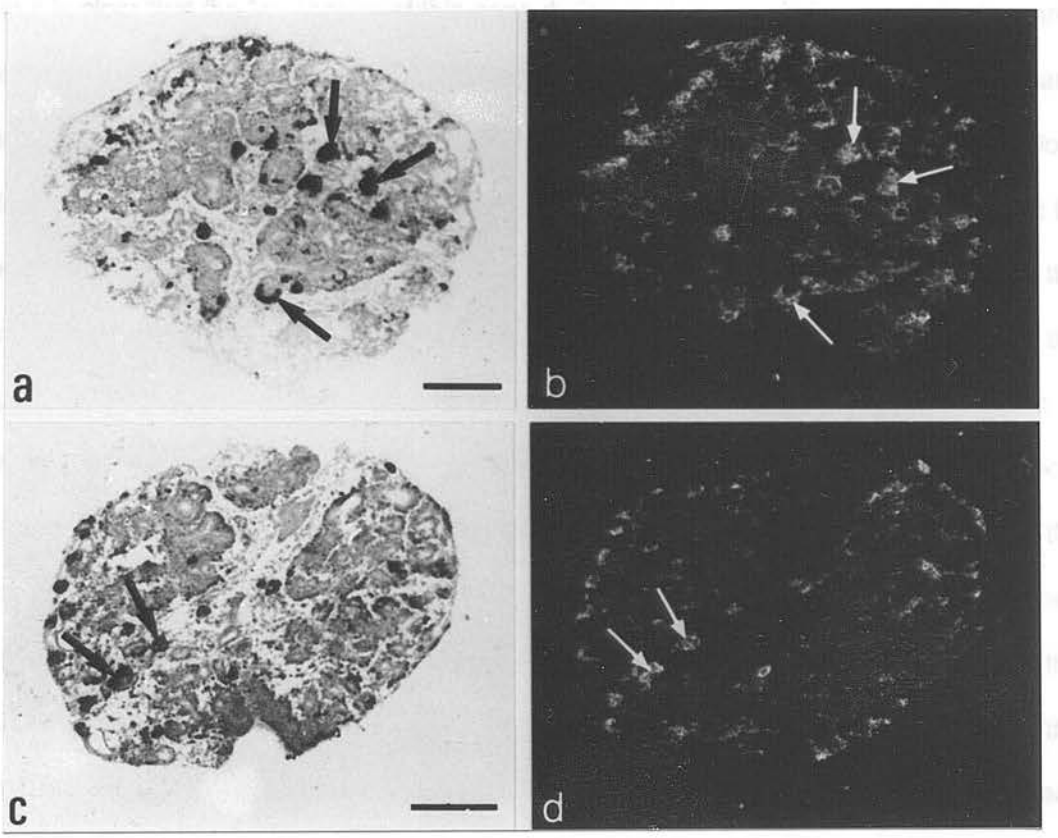



Figure 4.7. The effect of cytochalasin B on the expression pattern of *WT1* in culture (b,d, dark-fields). a) A control rudiment cultured for 6 days, demonstrating that the developing nephron (arrows) expressed the gene at a higher level than the condensed mesenchyme surrounding the branches of the collecting system (bar: 200 μm). c) A section of a rudiment cultured for 6 days in medium that contained 0.2 μg/ml of cytochalasin B. Development had been affected slightly, so that fewer epithelial aggregates had formed (arrows; bar: 250μm).

## 4.3 Conclusions

In this chapter the pattern of *WT1* expression during the formation of the mouse metanephric kidney has been examined in detail. If we compare the *in vivo* results obtained here, with those previously published for the human embryo (Pritchard-Jones *et al.,* 1990), then it is clear that the localisation of this gene during nephrogenesis is essentially the same between the two mammalian species. One major difference has emerged, however, and this concerns the question of whether the metanephric mesenchyme expresses *WT1* before induction occurs. In contrast to the human fetal kidney, where *WT1* expression could not be demonstrated in uninduced tissue, it seems that the mouse metanephros does express the gene before induction. It cannot, however, be ruled out that this result reflects the experimental problems associated with demonstrating such a low level of expression in human embryonic material, rather than a real difference between the two species. In both species, it is clear that *WT1* is upregulated after induction and this result together with the increased level of expression in the forming nephron suggests that this gene is transcribed at three different levels in developing kidney. Finally, *WT1* mRNA was still detectable in the adult mouse kidney, albeit at a low level, where it was restricted to the podocyte cells of the renal corpuscle. Earlier reports had only examined metanephric tissue from the early postnatal period (Pritchard-Jones *et al.,* 1990; Pelletier *et al.,* 1991a), so this observation extends the previous understanding of the long-term maintenance of this gene.

An *in vitro* study using the whole-organ culture system was also presented in this chapter and has shown that the expression pattern of *WT1* is the same as that seen *in vivo.* This will be of use when the other genes implicated in WT are isolated, because it will enable their temporal and spatial interaction with *WT1* to be investigated in a system that can be experimentally controlled. The *in vitro* system has also allowed the effect of manipulating the process of nephrogenesis on the pattern of *WT1* expression to be investigated and in particular has shown that cytochalasin B and LIF exert their effect downstream of induction.

The implications of these results together with those obtained in the *in vivo* study will be not be considered here, but will be examined in more detail in the final chapter.

As the pattern of *WT1* expression is very similar in the human and mouse kidneys, it is curious that the later does not develop WT. In an attempt to examine this further, a model that had previously been proposed to produce these tumours in the mouse (Javadpour & Bush, 1972) has been studied and the results are presented in the following chapter.

# CHAPTER 5

# INVESTIGATING A POSSIBLE MOUSE MODEL OF WILMS' TUMOUR

## 5.1 Introduction

The inherent problems associated with studying human embryonic and tumour material mean that an animal model of WT would greatly facilitate the study of both the malignancy itself and the role of *WT1*. Unfortunately, the spontaneous occurrence of nephroblastoma in species other than man is extremely rare (Gonzalez-Crussi, 1984; Hard, 1984a,b), with there being only one uncorroborated report in the mouse (Guerin *et al.*, 1969).

Although a genetic model of the disease, the *Small-eye* (*Sey*) mutant has been described (for details, see chapter 1), the only system put forward that proposes to produce the WT phenotype in the mouse has given inconsistent results. This system was first described by Javadpour & Bush (1972) who reported that, if embryonic kidney fragments were placed under the testis capsule of adult syngeneic mice, large tumours formed that had Wilms' morphology and that gave rise to metastases. A re-examination of the model found, however, that the transplanted tissue developed normally, albeit at a delayed rate, and could find no evidence of metastases (Mount *et al.*, 1974) .

With the intention of resolving the usefulness of this system, the response of a range of mouse strains to the insertion of metanephric rudiments has been investigated and the results are presented in this chapter. The adult kidney was used as an additional host site and this allowed the dependence of the response on the sex of the host to be analysed for the first time. It is of interest, in this context, that the embryonic kidney rudiment has been shown to give rise to new functional nephrons when inserted into the cortex of an adult kidney (Woolf *et al.*, 1990). The growths obtained in this study were examined both for their basic morphology and for the degree of their molecular differentiation using antibodies to developmental markers in conjunction with *in situ* mRNA hybridisation analysis with the mouse *WT1* gene. In this chapter the results will be described and the implications for this system in the further study of kidney disease will be considered.

## 5.2 Results

### *5.2.1 The formation of growths*

The strain of mouse used in the original studies, the 129/Sv, is prone to spontaneous testicular teratomas (Lyon & Searle, 1989). In order to minimise the effect that this might have had on the results, the outbred Swiss mouse strain was used in a preliminary study. The results from this initial work did not, however, replicate those of the original study and this led to the use of both the 129/Sv and another inbred strain, the CBA, so enabling the strain dependence of the response to be investigated.

Figures that summarise the numbers of growths obtained and their morphology are given in Table 5.1. The overall percentage of growths (64%) agrees with that of Javadpour & Bush (1972), but more were recovered from the kidney (75%) than the testis (45%). This observation can probably be accounted for by the fact that, while rudiments inserted under the kidney capsule could be seen clearly, those within the testis could not. It was not therefore possible to ensure that all of the rudiments in the latter case had been successfully transferred. In addition, more growths were obtained from the experiments involving 12.5 and 13.5 day rudiments than from the 14.5 day tissue, with only 7% of the growths falling into this latter group.

The growths reported by Javadpour & Bush (1972) had obtained a size of greater than 1 cm in diameter within 4-6 weeks. In contrast, those obtained in this study, which were initially about 0.8 mm in diameter (Figure 5.1a), had reached their maximum of 2-5 mm after 8 weeks (Figure 5.1b). Many of them showed clear signs of vascularisation, but all were easily removable from the host tissue and showed no evidence of local invasiveness.

In order to investigate whether any of the growths gave rise to metastases, the carcasses were all examined superficially for evidence of abnormality. In addition, several of the mice were given detailed autopsies and in each case their peri-aortic lymph nodes were found to be normal. Two of the 129/Sv mice that had been given kidney operations developed

Table 5.1. The number of growths and their morphologies, for the 3 strains of mice.

| strain | host site | total no. transferred | no. growths obtained | % success of growth development | blastemal cells | multiphasic | cystic |
|---|---|---|---|---|---|---|---|
| Swiss | kidney | 14 | 10 | 71 | 1 | 8 | 1 |
| | testis | 12 | 4 | 33 | - | 2 | 2 |
| CBA | kidney | 20 | 17 | 85 | - | 1* | 16 |
| | testis | 14 | 6 | 43 | - | - | 6 |
| 129/Sv | kidney | 22 | 15 | 68 | 3* | 6** | 6 |
| | testis | 5 | 4 | 80 | - | - | 4 |

* this had cysts

** four had cysts

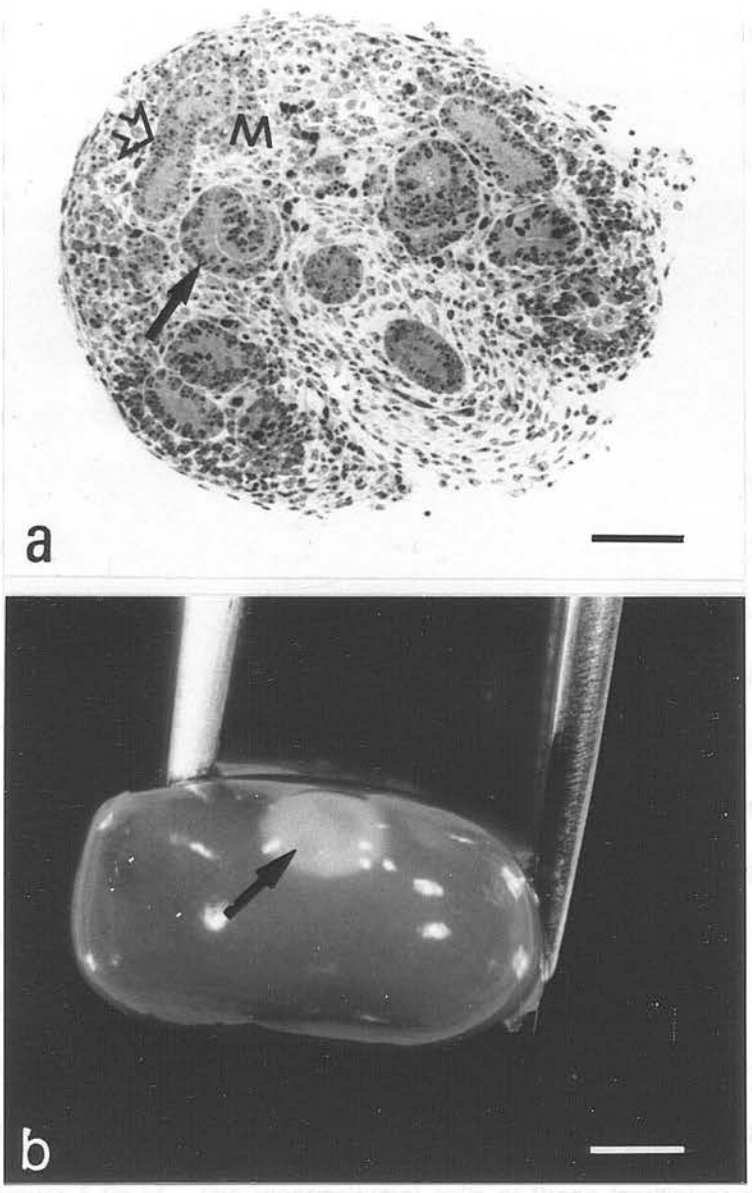



Figure 5.1. a) A section of a 13.5 day mouse kidney showing part of the bifurcating ureteric bud (hollow arrow) with an associated cap of condensed metanephric mesenchyme (M) and a comma-shaped stage (solid arrow; bar: 100 μm). b) The kidney from an adult Swiss mouse demonstrating the 10 week growth (arrow) that formed following the insertion of a 12.5 day rudiment under the capsule (bar: 2 mm).

large testicular tumours, but owing to the susceptibility of these mice to such tumours, this was considered to be irrelevant to the study.

The morphology of the growths seemed to be independent of both the time spent *in situ* (for details, refer to section 5.2.2) and of the type of host tissue (see Table 5.1). Major strain differences were, however, observed from the analysis of the sectioned material and each of these will now be considered in turn.

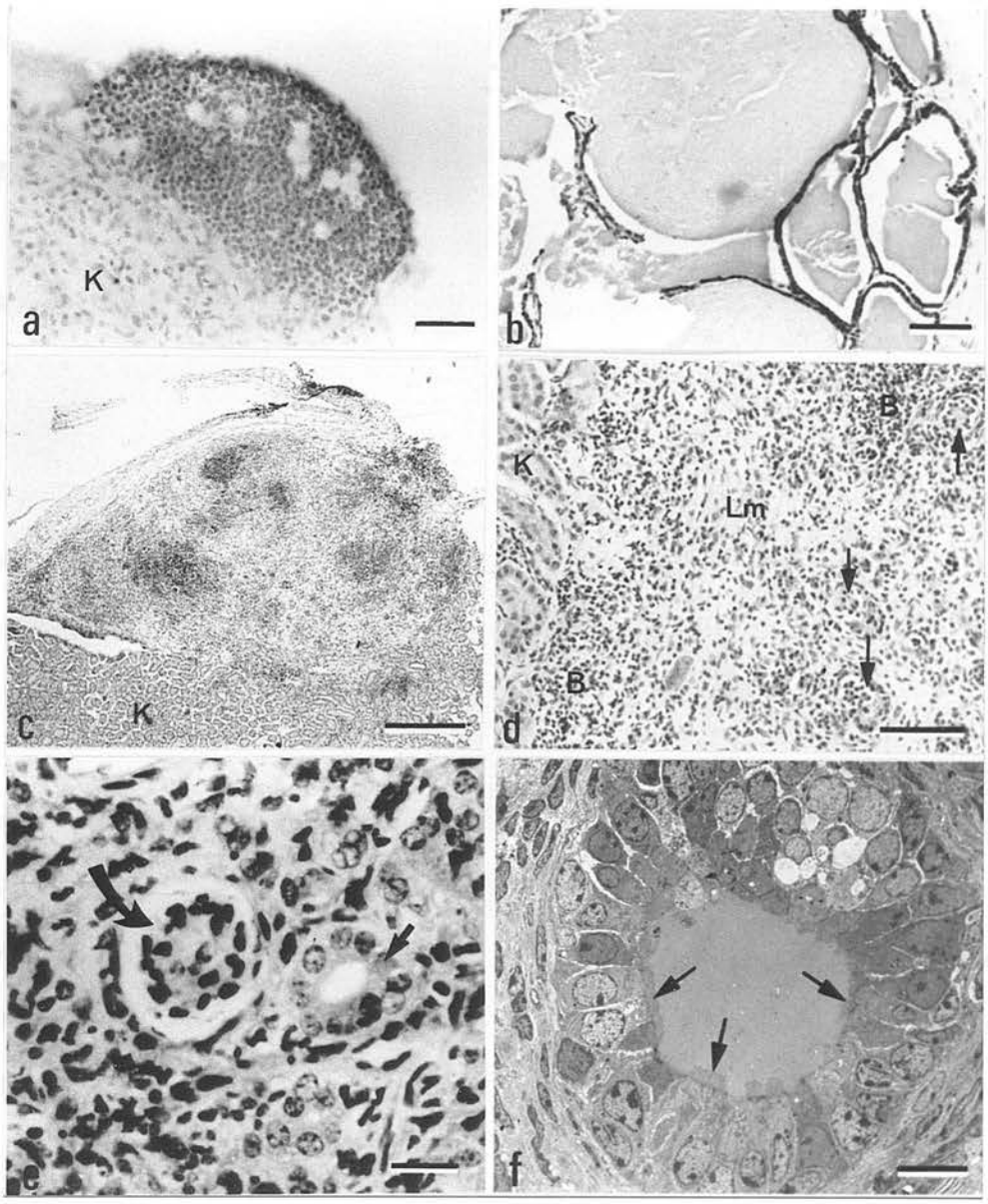
## *5.2.2 Strain-dependent morphology*

### 5.2.2.1 Swiss mice

Three distinct morphologies were observed in the 14 growths examined from this strain of mouse. The implanted tissue was removed after a period of between 5-15 weeks, with the average length of time being 9 weeks. One growth, removed after 5 weeks, was primarily composed of mesenchymal cells that contained little cytoplasm and resembled the blastemal component of WT (Figure 5.2a). Three more, which averaged 9.5 weeks, were found to comprise of epithelial components that had become cystic (Figure 5.2b): these were analogous to those seen in the CBA strain and this morphology is considered in more detail later. The remaining 10 growths resembled those of Javadpour & Bush (1972) and had many of the histological characteristics of classic triphasic WT.

This third class of growths contained large areas of mesenchymal cells that included randomly distributed epithelial structures and was characterised by a lack of cellular organisation (Figure 5.2c-e). The mesenchymal cells of these 'multiphasic' growths had two distinct morphologies; those with very little cytoplasm, that resembled blastemal cells and those with more cytoplasm that had the appearance of the stromal component of WT (Figure 5.2d). An unexpected feature, however, was the observation of many glomeruloid-bodies (Figure 5.2d). Furthermore, examination of the epithelial components using the TEM, showed the presence of tubules with brush borders (Figure 5.2f). These resembled the proximal tubules of the adult kidney, with spherical nuclei situated basally and with numerous microvilli forming an apical brush border.

Figure 5.2. Morphology of the types of growths seen in the Swiss mouse. a) A section of a 5 week growth that formed when a 12.5 day embryonic kidney was placed under the adult kidney (K) capsule. It was composed entirely of mesenchymal cells, with prominent nuclei and little cytoplasm, that resembled the blastemal cells of WT (bar: 50 μm). b) A growth formed after a 12.5 day rudiment was inserted under the adult kidney capsule and left in place for 10 weeks. Large fluid-filled cysts had formed that were lined with epithelial cells (bar: 100μm). c) A section through an 8 week growth that formed when a 13.5 day rudiment was inserted under the adult kidney (K) capsule (bar: 0.5 mm). d) The same section at higher power, illustrating the multiphasic morphology that was characterised by areas of blastemal cells (B) and loose mesenchyme (Lm). In addition, there were many glomeruloid-bodies (arrows; bar: 100 μm). e) At a higher magnification, a glomeruloid-body (left) and an epithelial tubule (right) were evident in the same section (bar: 20 μm). f) A TEM micrograph of a 10 week growth, formed when a 12.5 day rudiment was inserted into the adult kidney and showing a tubule with a well-defined brush border, an indicative feature of the proximal tubule (bar: 10 μm).


a
K
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K
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K
Lm
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B
e
f

Primitive epithelial elements are often observed in WT, but glomeruloid-bodies are less common (Payton *et al.*, 1988) and the inclusion of large numbers of such structures in themultiphasic growths was the first suggestion that they had undergone a degree of differentiation inappropriate for a true model of WT. This was confirmed by the demonstration of proximal tubules, which differentiate relatively late in nephrogenesis (Ekblom *et al.*, 1981a) and are rarely seen in WT (Mierau *et al.*, 1987).

#### 5.2.2.2 CBA mice

Of the 23 growths studied in CBA mice (8-26 weeks; average 17 weeks), one had the disorganised morphology characteristic of the Swiss strain (24 weeks, Figure 5.3a), while the rest contained large numbers of glomeruloid-bodies associated with, but seemingly unconnected to, very large cysts of up to 1 mm in diameter (Figure 5.3b,c). The majority of these cysts had a clearly defined epithelial lining and contained acellular material that had sometimes become crystalline. A small number of mesenchymal cells could be seen sandwiched between the epithelial structures and these resembled the stromal-type cells observed in the multiphasic growths.

Examination of the cystic growths using the TEM showed that the ultrastructure of the glomeruloid-bodies was similar to the renal corpuscle of the adult kidney (Figure 5.3d). The visceral and parietal layers of Bowman's capsule seemed to be normal and had well-formed podocytic processes enveloping the capillaries. This last observation is important, because although it was not possible to investigate further, it does indicate that vascularisation of these growths had occurred.

#### 5.2.2.3 129/Sv mice

The complete range of morphologies was observed in this strain, with a total of 19 growths remaining *in situ* for between 9 and 22 weeks (average 13 weeks; Figure 5.4). Of these, 3 contained purely blastemal cells (average 12 weeks), 6 had the disorganised morphology of the Swiss strain (average 14 weeks) and 10 had the cystic morphology

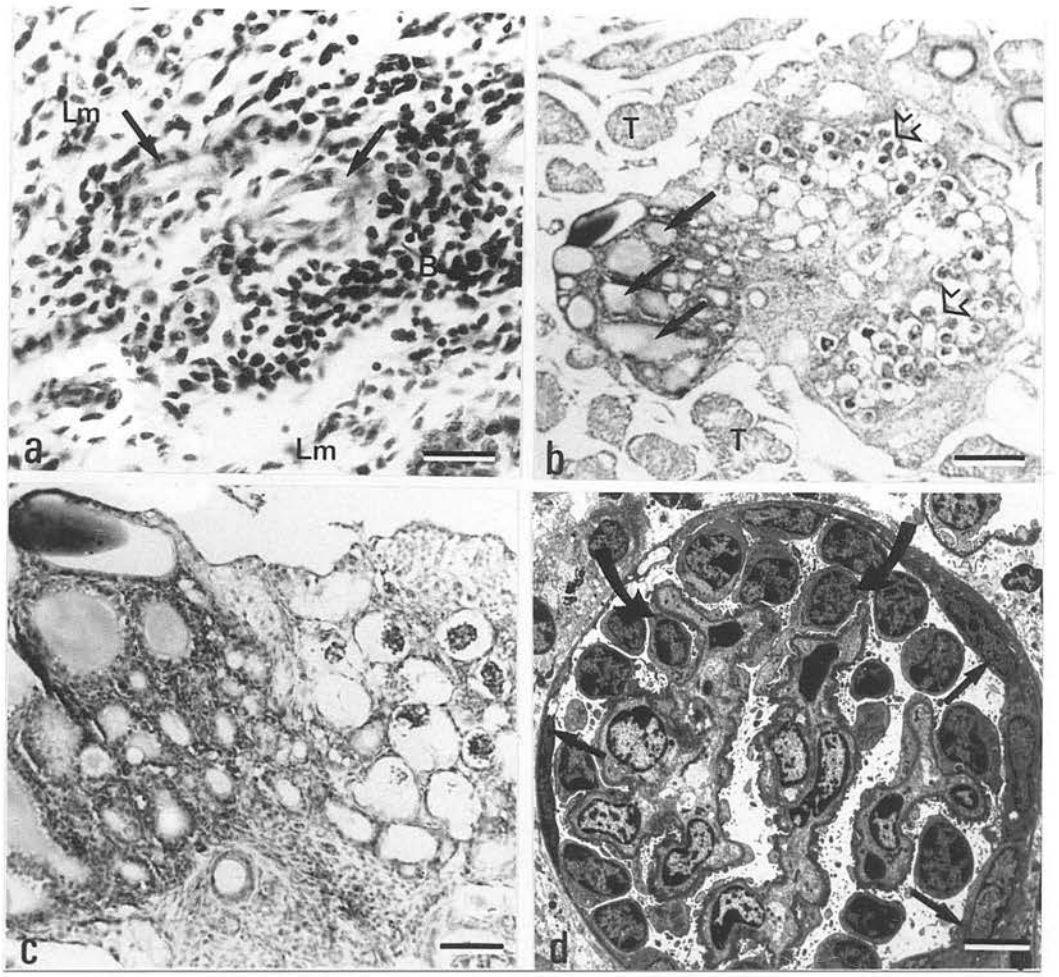
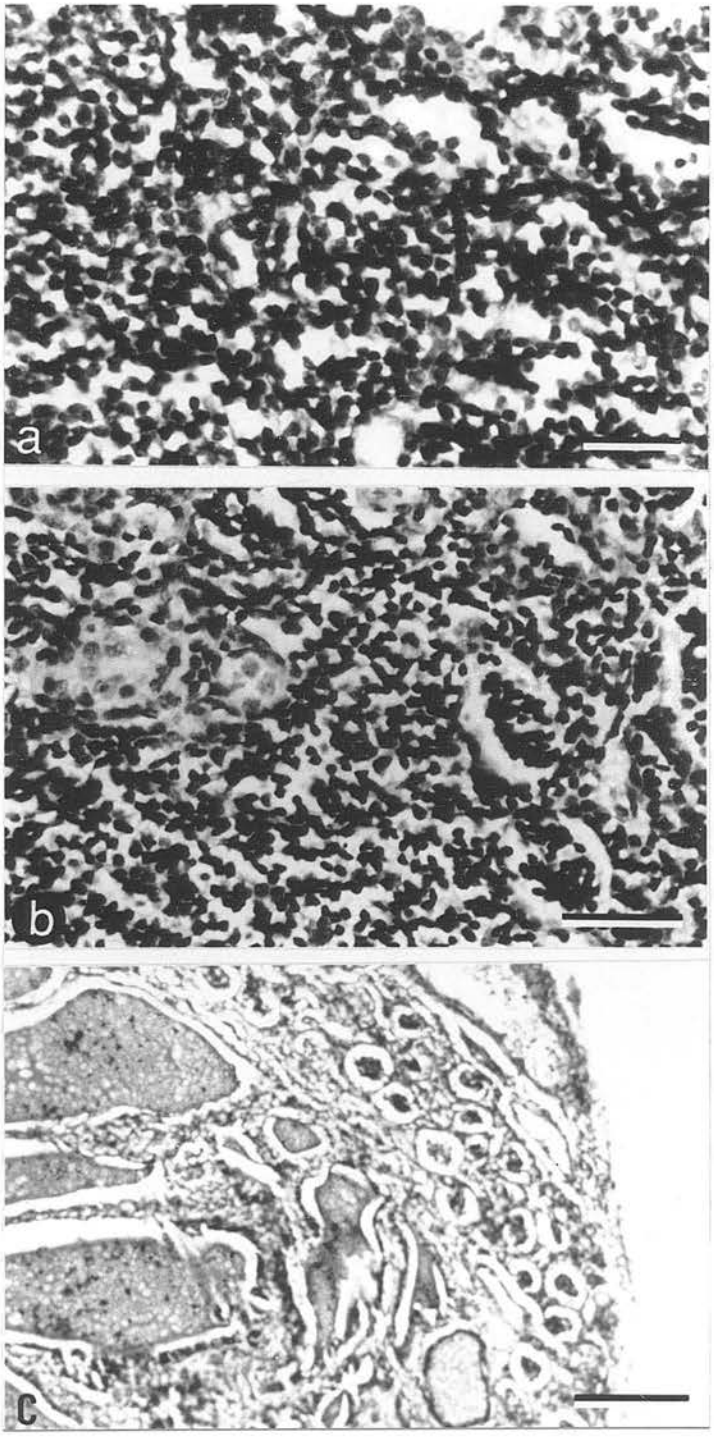



Figure 5.3. Morphology of the types of growths seen in the CBA mouse strain. a) A section of a multiphasic growth that formed when a 13.5 day embryonic kidney was inserted underneath the kidney capsule and left *in situ* for 26 weeks. Simple epithelial tubules (arrows) and mesenchymal cells resembling the blastemal (B) and stromal (Lm) components of WT were a characteristic feature of this type of growth (bar: 30 μm). b) A section of a 15 week growth that formed after a 12.5 day rudiment was inserted into the testis (T). This type of growth often showed a high degree of organisation, with areas of glomeruloid-bodies (hollow arrows) separated from the cystic elements (arrows; bar: 200 μm). c) The same section at higher power, illustrating the distended glomeruloid-bodies and fluid-filled cystic elements (bar: 100 μm). d) A TEM micrograph of a glomeruloid-body from a 7 week growth that formed when a 13.5 day rudiment was inserted into the adult kidney. The visceral (curved arrows) and parietal (arrows) layers were similar to those seen in the Bowman's capsule from a normal adult kidney (bar: 5 μm).

Figure 5.4. Morphology of the types of growth observed in the 129/Sv mouse. a) A section of a 12 week growth, composed entirely of blastemal cells, that formed when a 12.5 day embryonic kidney was inserted underneath the kidney capsule of an adult female mouse (bar: 30 μm). b) A growth that developed after 10 weeks, when a 13.5 day rudiment was transplanted underneath the kidney capsule of an adult female, showing the characteristic features of a multiphasic growth (bar: 50 μm). c) In a male mouse, a 13.5 day embryonic kidney inserted under the kidney capsule, produced a growth with cystic morphology after 10 weeks (bar: 150 μm).


a
b
c

previously described in the CBA strain (average 13 weeks). In this strain of mouse, one third of the animals used were female, but there was no obvious difference between the sexes in the morphologies of the growths that formed (e.g. Figure 5.4a,b).

### *5.2.3 The expression of developmental markers*

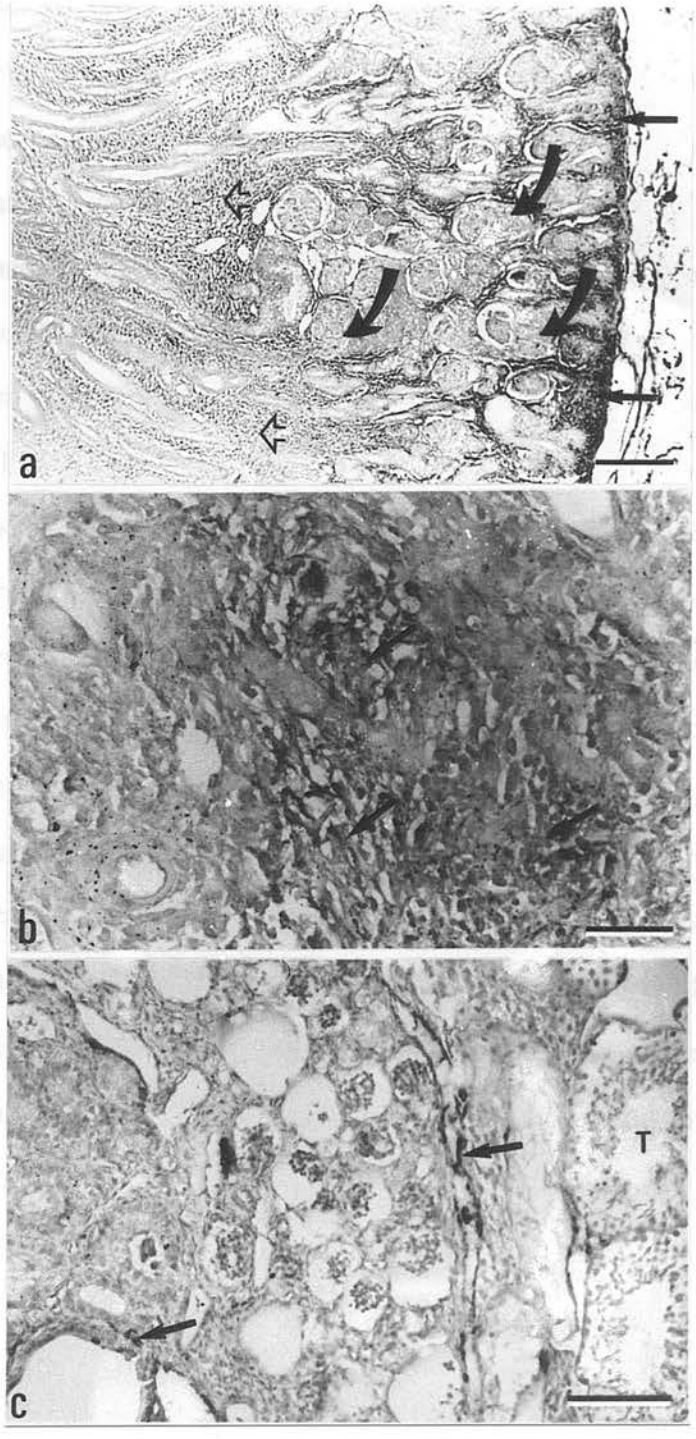
In order to clarify the histological observations, immunohistochemistry was carried out on a range of growths from the two main morphological categories. A monoclonal antibody that recognises the brush border of the rat proximal kidney tubule (BB110; Miettinen & Linder, 1976) and cross-reacts with the same segment of the mouse nephron (Ekblom *et al.,* 1980a) was used. To complement this epithelial marker of late nephrogenesis, a novel monoclonal antibody WT1.27, that had been developed in the MRC Human Genetics Unit, was used during this study. This antibody is known to react with a range of both human and mouse mesenchymal tissue, including the stem cells and differentiated mesenchyme of the kidney, but fails to recognise either induced mesenchyme that is undergoing nephrogenesis or any other epithelial tissue (Figure 5.5a).

In the multiphasic growths, the BB110 antibody reacted with a small number of epithelial tubules, while WT1.27 stained the extensive areas of mesenchymal cells seen in this category (Figure 5.5b). The cystic growths showed restricted staining with both antibodies, with only a subset of the dilated tubules being positive for BB110 and very little evidence of WT1.27 reactivity (Figure 5.5c). These results confirm the histological analysis, that both types of growth had differentiated to a level that was inappropriate for a model of WT. For the cystic growths, it was also possible to determine from this data that some of the cysts had formed within the proximal tubule of the nephron.

### *5.2.4 The expression of* WT1

In addition to immunohistochemistry, the two main morphological categories were examined for the expression of the WT predisposition gene, *WT1,* using *in situ* mRNA hybridisation. In the initial rudiments, the gene would have been expressed in the condensed

Figure 5.5. The staining pattern of the monoclonal antibody WT1.27. a) A section of a 17 week human fetal kidney, illustrating that the stem cells (arrows) and differentiated mesenchyme of the medulla (hollow arrows) expressed WT1.27, whereas the epithelium of the nephron (curved arrows) did not (bar: 200 μm). b) In a 13 week multiphasic growth, that developed following the insertion of a 13.5 day embryonic kidney into the testis of a Swiss mouse, extensive staining of the mesenchyme (arrows) was observed (bar: 40 μm). c) A section of a cystic growth, formed when a 13.5 day rudiment was placed in the testis of a CBA mouse and left *in situ* for 16 weeks, demonstrating that little staining (arrows) was observed in this class of growth (bar: 200 μm).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T

cap of mesenchyme surrounding the collecting system and in the pretubular aggregates, becoming confined to the visceral layer of Bowman's capsule as development proceeded (see chapter 4, for details). In WT itself, the gene is restricted to the blastemal cells and certain epithelial components, that include the glomeruloid-body (Pritchard-Jones & Fleming, 1991). The pattern of *WT1* expression was thus useful in two respects; to investigate the state of differentiation obtained in the growths and as a measure of their true value as a model for WT.

Examination of the multiphasic growths showed that, although the background was high on all the sections, no signal above this level was found in the mesenchymal cells (Figure 5.6a), including both those that resembled the blastemal component of WT and those that resembled the stromal cells. The epithelial tubules did not express *WT1*, but the glomeruloid-bodies showed a high level of expression, with the signal being confined to the visceral layer of Bowman's capsule in the more mature examples (Figure 5.6a). In the cystic growths, positive signal was again confined to the glomeruloid-bodies, with no expression above background level being observed in the mesenchymal cells or the cystic elements (Figure 5.6b).

The fact that the blastemal cells did not express *WT1* was unexpected in the light of the pattern of expression in the initial rudiments and in WT itself and points to the inadequacy of this system as a model for the tumour. If we consider the origin of these cells, it can be suggested that they were derived from three possible sources; the differentiated mesenchyme of the initial rudiment, the stem cells of the outer cortex or the condensed mesenchyme of the renal aggregates. In the first instance, the expression pattern of *WT1* can be easily explained because differentiated mesenchyme does not express this gene. For the other two, the situation is different as these cells normally express *WT1* and would have had to switch-off the transcription of this gene.

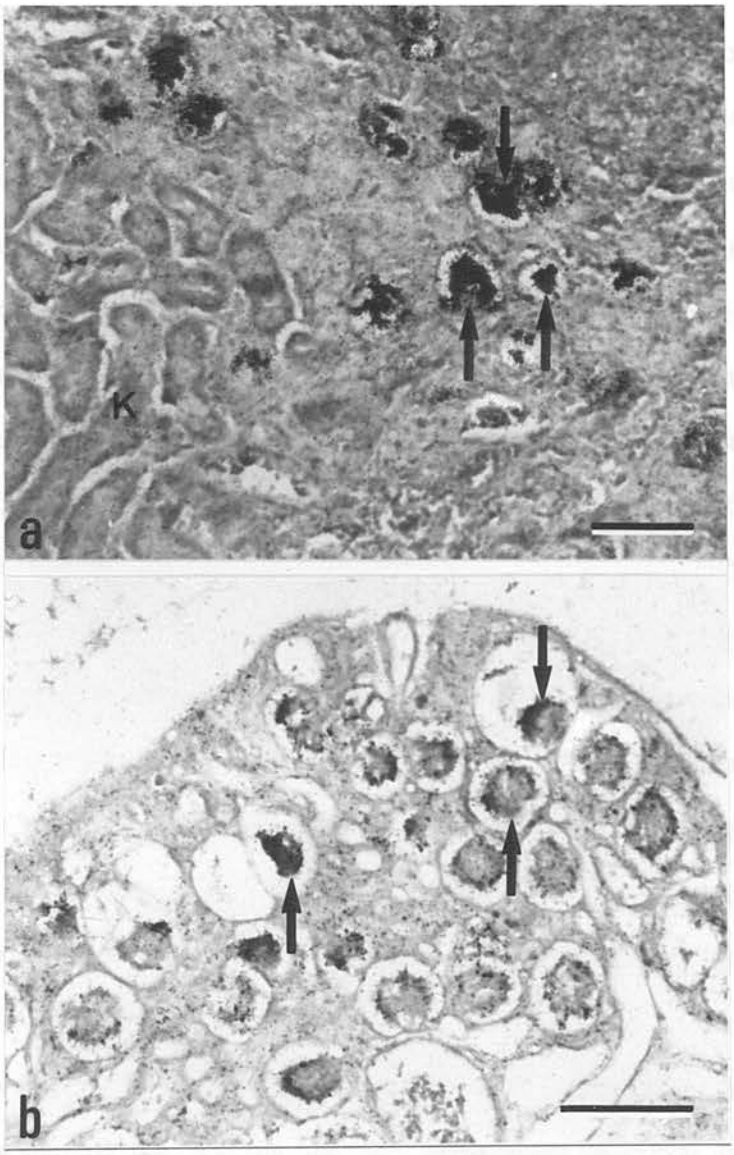


Figure 5.6. Expression pattern of *WT1* using *in situ* mRNA hybridisation. a) A section of a 10 week multiphasic growth formed after a 13.5 day embryonic kidney was placed under the capsule of the kidney (K) of an adult female 129/Sv mouse (adjacent section to Figure 5.4b). Silver grains indicating positive signal were restricted to the glomeruloid-bodies (arrows; bar: 100 μm). b) In a growth with cystic morphology that formed after 15 weeks in the testis of a CBA mouse following the insertion of a 12.5 day rudiment, labelling was again restricted to the glomeruloid-bodies (arrows; bar: 100 μm).

## 5.3 Conclusions

The results presented in this chapter show that the implantation of metanephric rudiments underneath the capsule of the testis and the kidney of adult syngeneic mice, leads to neither the malignant growth nor the development of metastases described previously (Javadpour & Bush, 1972). The rudiments did, however, grow to a certain extent in these ectopic sites and underwent varying degrees of differentiation that seemed to depend on the strain of mouse used. The potential of this system for modelling Wilms' tumour and its usefulness in the study of other aspects of kidney disease will now be considered.

Of the 3 classes of morphologies observed, the multiphasic growths were the most similar to the results from the original study (Javadpour & Bush, 1972) and superficially resembled triphasic WT. The demonstration, however, of many glomeruloid-bodies and epithelial elements together with results of experiments using developmental markers, leads to the conclusion that these growths had undergone a degree of differentiation inappropriate for WT. This observation was even more pronounced in the second class of growths, those with fluid-filled cysts. In contrast to the large numbers of glomeruloid-bodies and epithelial tubules that they contained, these growths had few mesenchymal cells. The third class of growths, those that resembled blastemal cells, seems to have arisen from cells that had neither differentiated nor divided, a result consistent with their having remained isolated from their normal inductive stimulus (i.e. see the transfilter experiments described in chapter 4). The expression pattern of *WT1* was particularly helpful in investigating how applicable this model was to the study of WT and it can be concluded that it is not a reliable method for the production of this tumour in the mouse. The strain variability in the morphology of the growths obtained is intriguing, but it is difficult to offer any explanation for this effect.

It is apparent that this study failed to reproduce the observations of Javadpour & Bush (1972), but the reasons for this also remain unclear. It has been suggested (Mount *et al.,* 1974) that the original tumours arose as a result of contamination of the metanephric rudiment with remnants of the genital ridge, which have been shown to produce tumours (Stevens, 1964).

Alternatively, the 129/Sv strain used here may have diverged slightly from the original one and given the strain variability in the growths produced in this study, this possibility cannot be excluded.

The consistent production of the cystic morphology by the CBA strain of mouse, on the other hand, indicates that this model may be of use in the study of polycystic kidney disease (PKD), a term that covers a variety of cystic renal disorders (reviewed by Potter, 1972; Crawfurd, 1988). Animal models do not yet exist for all the categories of this disease, although the C57BL/6J-*cpk* mouse develops cysts during embryogenesis that resemble those found in autosomal recessive PKD (Preminger *et al.*, 1982; Mandell *et al.*, 1983). In these mice, small dilations of the proximal tubules are seen in embryos and neonates, while the large cysts characteristic of the disease seem to be derived from the collecting system. Autosomal dominant PKD, the other major inherited form of the disease, also has a murine model (Takahashi *et al.*, 1991), although this has not yet been fully defined.

In order to investigate the experimental value of the CBA results further, the mechanism underlying cyst formation in these mice will need to be investigated. A first step in achieving this would be to determine whether ultrafiltration is occurring in this class of growth. FITC dextran, that has proved to be a good marker for this process (Woolf *et al.*, 1990), could be injected into the animals systemic system in order to test this hypothesis. Alternatively, biochemical analysis could be carried out on the fluid from the cysts and could then be compared to values obtained from the blood plasma and the urine. The second type of analysis would also enable the segment of the nephron that had become cystic to be examined.

**Addendum:** The results presented in this chapter have been accepted for publication by *Experimental Nephrology*.

# CHAPTER 6

# DISCUSSION

## 6.1 Introduction

A key area in the current study of development is the elucidation of the molecular controls that underpin cell differentiation and, with the isolation and characterisation of a variety of factors involved in committing a stem cell to a specific developmental pathway, we are beginning to understand the mechanisms underlying this process. The WT predisposition gene, *WT1,* recently isolated by positional cloning, is of particular interest here, because the evidence suggests that it normally plays such a role in kidney development, while its mutated form can lead to a tumour. Such a gene should allow the relationship between abnormal differentiation and malignancy to be investigated.

It is within this context that the work presented in thesis should be examined. The expression pattern of the mouse homologue of *WT1* has been investigated in three complimentary systems; the developing embryo, the metanephric kidney both *in vivo* and *in vitro* and a proposed mouse model of WT. In undertaking such a study, the principal aim was to determine the extent of *WT1* expression with the intention of clarifying its role in normal and aberrant development, with a secondary aim emerging during the course of this work which was to examine the use of this gene as an experimental assay of kidney development.

The results underpinning this work come from the human embryo (Pritchard-Jones *et al.,* 1990), where it was shown that the renal system, the gonads and the mesothelial tissue of the coelomic cavity, all expressed *WT1.* By examining the mouse, it has been possible to extend these observations both temporally, to include the earliest point of expression, and spatially, to include several additional tissues. The key results from this study have been detailed in each experimental chapter, but here the data will be discussed in more depth, with the scope for further experimental work being considered at the end of the chapter.

## 6.2 The role of *WT1* in non-nephrogenic mouse development

The limitation of *WT1* transcription to a specific set of tissues is in sharp contrast to *RB1*, the paradigm of tumour suppressor genes involved in embryonal malignancies. *RB1* is expressed ubiquitously during mouse embryogenesis (Bernards *et al.*, 1989), while homozygous inactivation of this gene leads to a primary tumour in a specific tissue, the retina of the eye (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987). The restricted pattern of *WT1* expression in the human embryo (Pritchard-Jones *et al.*, 1990) had suggested three related and overlapping developmental roles for the gene; in the general mesenchyme-to-epithelium transition that is undergone by several of the tissues within the coelomic cavity, in the formation of the urogenital system and in nephrogenesis. The results obtained in this mouse study need to be considered for each of these in turn: the first two examples will be examined here, with nephrogenesis being discussed in a later section. In addition, three sites of *WT1* expression that do not fit into any of these categories will also be considered.

### *6.2.1 The mesenchyme-to-epithelium transition*

The transformation of mesenchymal cells to epithelium is relatively rare and has received little attention in the literature of development. The one obvious exception is the metanephric kidney and this has been studied extensively: here, a reciprocal inductive interaction between the ureteric bud and the mesenchyme leads to the latter forming the nephrogenic epithelium. In addition to the kidney, *WT1* is expressed in the mesothelium, the epithelium that lines the coelomic cavity and tissues within it, which derives from lateral plate mesoderm. The gene is also transcribed in the spleen and parts of the gonads that are thought to arise in turn from the mesothelium.

There are, however, several well-known tissues undergoing this transition where the gene is not expressed during mouse development. These include the corneal endothelium, the epithelium at the inner layer of the cornea, which forms from neural-crest-derived

fibroblasts (Johnston *et al.*, 1979; Bard & Kratochwil, 1987); the endothelial cells of blood vessels, which are, of course, a subset of the epithelia and differentiate from splanchnic-mesoderm-derived angioblasts (Balinsky, 1965); the mesonephric duct that forms from lateral mesoderm (Poole & Steinberg, 1984) and the somites which go through a mesenchyme-to-epithelium transition during their morphogenesis (Duband *et al.*, 1987). In addition, the mesothelial lining of the coelomic cavity has differentiated before *WT1* transcription is initiated and the gene clearly has no role in this tissue at this time. Furthermore, the adrenal cortex, that does not express *WT1* at any stage of its development in the mouse embryo, has been suggested to arise from the same origin as the spleen and the somatic cells of the gonads (Gruenwald, 1942), both of which express the gene.

The role for *WT1* in this general class of cell-state transitions therefore seems to be more limited than has previously been suggested. The tissues that express the gene may, however, have a more subtle connection. In both the mouse and human embryo, *WT1* is expressed in the mesenchyme underlying parts of the parietal layer of the mesothelium and an answer could lie in the nature of this tissue. This point was considered as long ago as 1942 by Gruenwald, who wrote:

*'It has long been known that the lining of the coelomic cavities differs from other epithelia during early periods of development by its close structural and genetic relations to the underlying mesenchyme. A persistence of the respective potencies permanently distinguishes the coelomic epithelium from other tissues of a similar structure.'*

A similar property of persistence has been attributed to the podocyte cells of the renal corpuscle, that are the only part of the nephron to express *WT1* after the renal vesicle stage and continue to do so long after development of the kidney has ceased. These cells form a semi-permeable filtration barrier and do not constitute a true epithelium, losing many of their epithelial characteristics as they develop (Garrod & Fleming, 1990). It is possible that *WT1* is involved in the subset of tissues that undergo a mesenchyme-to-epithelium transition, but retain certain mesenchymal qualities, although it is clear that until more is understood about the nature of this process it will be difficult to determine the exact role of the gene.

Nevertheless, as the gene is expressed in the mesothelium, it may be worth following the analogy with *WT1* expression in both the kidney and in WT and look to see if it is also transcribed in mesotheliomas. Second primary tumours are rarely associated with WT, but a number of those that have been reported were mesotheliomas (Austin *et al.*, 1986). This would not be without precedent, because in RB the most common second primary tumour is osteosarcoma (Hawkins *et al.*, 1987) and mutations in the *RB1* gene have been demonstrated in several of these cases (Friend *et al.*, 1986).

### *6.2.2 The development of the urogenital system*

In the case of the urinary and genital systems, there is strong genetic evidence to support a functional role for *WT1* and this coupled to their common developmental origin and the high level of urogenital anomalies associated with WT, has led to the hypothesis that the gene has pleiotropic effects on these systems (van Heyningen *et al.*, 1990). Additional evidence comes from the analysis of the expression pattern of *WT1* in the human fetus, with the gene being transcribed during the development of both the kidneys and the gonads (Pritchard-Jones *et al.*, 1990). Similar studies have been carried out for the mouse genital system (Pelletier *et al.*, 1991a; Pritchard-Jones, 1992) and the kidney (considered in section 6.3) and these confirm the data from the human.

The expression pattern of *WT1* in the early mouse embryo provides further proof to support this hypothesis. Transcription of this gene was observed in the urogenital ridge and overlying coelomic epithelium at a stage prior to any evidence of the differentiation of the genital component. Our failure, however, to detect *WT1* expression in the intermediate mesoderm of the 8 day embryo, the tissue that gives rise to the urogenital ridge, suggests that this gene is not involved in the earliest stages of urogenital development.

To digress slightly, the pattern of *WT1* expression in the early mouse embryo is interesting in the context of the evolutionary development of the urogenital system. The formation of the renal system in particular, embodies the idea that 'ontogeny recapitulates phylogeny', with the permanent kidneys of the more primitive vertebrate classes being

represented during the embryonic development of the amniotes as the transient pronephros and the vestigial mesonephros. The expression of *WT1* in the early embryo is restricted to the coelomic epithelium and the urogenital ridge and it is worth noting that in the lower non-amniotic vertebrates, the external glomeruli of the pronephros arise from the coelomic epithelium, while in all vertebrates the genital ridges develop from a thickening of this same epithelium (Kent, 1987). Furthermore, *WT1* has recently been isolated from *Xenopus laevis* (Jill Kent, personal communication), indicating that this gene is conserved between classes of vertebrates that are thought to have diverged more than 350 million years ago (McFarland *et al.*, 1979). Together these two lines of evidence highlight the fact that this gene might have had an important role throughout the evolution of the urogenital system.

### *6.2.3 Other sites of* WT1 *expression*

If we accept that *WT1* is involved in the mesenchyme-to-epithelium transition, then a second unrelated role is implicated by the demonstration that at least three tissues outwith this group express the gene; the cells that are undergoing differentiation into the skeletal muscle of the body-wall, a narrow domain within the spinal cord and a small region of the 4th ventricle of the brain. The transient expression of *WT1* during a specific stage of skeletal muscle development is of interest because heterologous elements such as muscle cells are observed in some cases of WT (Mierau *et al.*, 1987).

It is hard to see what part *WT1* plays in the differentiation of neural tissue, because the specific cells where the gene is expressed do not have a known function. *WT1* was first transcribed in a small domain of cells at the ependymal-mantle border, before becoming restricted to the ventral horn region of the mantle layer from about day 13 of gestation. Whilst it is known that the mantle layer goes on to form the grey matter of the spinal cord and the ventral horn region contains the cell bodies of the motor neurons, it is difficult to be any more definite. It seems unlikely, as has been suggested in the case of the human embryo (Pritchard-Jones, 1992), that *WT1* is expressed by the motor neurons that innervate the limbs, because although there was an elevated level of signal in the region of the

hindlimb buds, an increase in the corresponding cervical expansion, that innervates the forelimbs, was not observed.

While little is known about the mechanisms responsible for motor neuron differentiation in the spinal cord, it is worth pointing out that other genes involved in transcriptional regulation are also expressed within similar domains. Like *WT1,* but slightly ventral to it, the homeobox-containing gene, *Hox-8,* is expressed in narrow domain on the ependymal-mantle border (Duncan Davidson, personal communication), while the engrailed gene, *En-1,* and the paired-box gene, *Pax-2,* are expressed over more extensive areas (Davidson *et al.,* 1988; Nornes *et al.*, 1990). The fact that at least 4 genes with roles in development are specifically expressed within a similar region of the spinal cord (they all lie within the area of AP activity that marks lateral motor column formation) indicates the need to investigate the functional significance of this domain further and suggests that there may be a connection among this group of transcription factors. Another point worth making is that division of the motor neurons has ceased by day 11 of mouse gestation and they are in fact being reduced in number by programmed cell death. This invites comparison with another homeobox gene, *Hox-7.1*, that is expressed in the mouse limb in the regions between the digits where cell death is known to occur (Hill *et al.,* 1989).

A degree of caution should, however, be employed when suggesting a functional role for a particular gene based on its pattern of expression alone. In a recent study, gene targeting in ES cells was used to disrupt the homeobox containing gene *Hox-1.5* and mice were then produced that were homozygous for this mutated locus (Chisaki & Capecchi, 1991). The phenotype of these animals showed that the genetic function of *Hox-1.5* is more restricted both temporally and spatially (Chisaki & Capecchi, 1991) than had been predicted by studies of gene expression using *in situ* mRNA hybridisation (Gaunt, 1987; 1988).

## 6.3 The role of *WT1* in nephrogenesis

The involvement of *WT1* in normal kidney development has been of particular interest because mutations in this gene are thought to block the process and can lead to the formation of WT. Three separate lines of research indicate that the *WT1* gene product, a protein with 4 zinc fingers, has an important function in nephrogenesis. First, the *WT1* locus is sometimes deleted in individuals with the rare WAGR syndrome, a constellation of anomalies that includes WT and malformations of the renal system. Second, the expression pattern of the gene in the human fetal kidney is consistent with it having a functional role during the normal development of this organ. Finally, high-affinity binding sites for the WT1 protein have been found in the promoters of several genes that are known to be involved in nephrogenesis.

The evidence presented here emphasises this second point: not only is the pattern of *WT1* expression during the development of the mouse kidney similar in most respects to that previously described for the human embryo (Pritchard-Jones *et al.*, 1990), but several additional points have also emerged. These allow us to re-evaluate the normal role of this gene and in particular its involvement in induction.

The reciprocal inductive interaction between the ureteric bud and the metanephric mesenchyme is the key event underpinning the formation of the kidney. The changes that occur in the mesenchyme following induction have been studied extensively and it seems evident that this process initiates the production of factors that in turn activate others and leads to a cascade that controls the pattern of differentiation (see chapter 1, for details). A recent study has shown that the pleuripotency of the stem cells is restricted some time after induction, so that a cell contributes to only a single segment of the nephron (Herzlinger *et al.*, 1992). Little is known, however, about the mesenchyme before induction takes place, although it is recognised as being already committed to becoming nephrogenic tissue (Saxen, 1970).

The observation that *WT1* was expressed in the mouse before induction at a level that increased as a direct result of this interaction indicates that the gene plays an important part in the process itself. The data is consistent with the idea, which has already been proposed (Pritchard-Jones *et al.*, 1990) and will be considered in more detail later, that the normal function of WT1 is to regulate the transcription of genes involved in cell proliferation and/or differentiation, thus playing a key role in initiating the cascade of nephrogenic development.

We first need to examine the implications of *WT1* transcription occurring before induction, albeit at a low level. A possible suggestion is that expression of the gene is essential for competence. It should, however, be pointed out that expression was first detected at a time when the metanephric mesenchyme could already be distinguished from the surrounding cells as a discrete group surrounding the ureteric bud, so indicating that other factors are involved in assigning a metanephric identity to this particular part of the intermediate mesoderm.

An important question that has not yet been properly addressed is whether all of the metanephric mesenchyme is induced in the initial interaction with the ureteric bud. There are two lines of evidence that point to this being the case, for the mouse at least. The *in vitro* study demonstrated that the level of *WT1* transcription in isolated mesenchyme increased uniformly throughout the tissue if it was cultured with an inducer for 24 h. In addition, *in vivo* analysis of 12 and 13 day embryonic kidneys has shown that all of the undifferentiated stem cells in the outer cortex of the organ, expressed the gene at a level compatible with induction having taken place. Furthermore, the demonstration that LIF reversibly blocks nephrogenesis at a point just downstream of induction (see section 6.5) confirms the hypothesis that this molecule, or one like it, could provide the restriction that would be needed *in vivo* to allow induced stem cells to enter the nephrogenic pathway of differentiation at a controlled rate (Bard & Ross, 1991).

If we accept the idea that all the mesenchyme is induced during its initial interaction with the ureteric bud, then we can answer a second question: is the interstitial mesenchyme

of the kidney derived from mesenchyme that is induced? This problem has already been examined by Pritchard-Jones & Fleming (1991), who proposed two models based on their results from human embryonic and tumour tissue. In the first of these, some of the cells of the condensed mesenchyme are excluded from the renal vesicle by the formation of a continuous basement membrane and these cells revert back to a mesenchymal phenotype. In the second model, the interstitial mesenchyme is suggested to arise directly from the uninduced mesenchyme that has not undergone condensation. Our results predict that the interstitial mesenchyme is derived from cells that have been induced. This does not, however, exclude the second model if it is modified slightly, so that the differentiation pathway of the interstitial mesenchyme diverges immediately after, rather than before, induction.

In terms of suggesting a functional role for WT1, the observation that the gene is transcribed at three distinct levels in the mouse, is of obvious importance. Having considered the first of these, let us now move on to discuss that seen in the induced mesenchyme, where WT1 may well initiate the cascade of gene activity that leads to nephrogenesis taking place. It seems unlikely that WT1 acts at this stage to repress the transcription of genes involved in cell proliferation, because this is the initial reaction of the metanephric mesenchyme to induction (reviewed by Saxen, 1987). A more conceivable role is the activation of genes involved in cell adhesion, such as syndecan, which are an early response to induction and lead to the condensation of the metanephric mesenchyme (Vainio *et al.*, 1989).

The third level of *WT1* mRNA was seen in the renal vesicle, which is undergoing the mesenchyme-to-epithelium transition. WT1 could be involved at this stage in regulating the transcription of a number of genes, including switching off those involved in cell proliferation, such as *IGF-II*, or conversely activating those that specify the epithelial phenotype (see chapter 1, for details). It is intriguing that the gene is switched off in the parts of the nephron destined not to contribute to the renal corpuscle, but continues to be transcribed into adulthood in the podocyte cells, albeit at a reduced level. This observation has already been

considered in section 6.2.1, with the suggestion that *WT1* is involved in maintaining the specialised nature of the podocyte cells.

Evidence is beginning to accumulate about the downstream targets of WT1 *in vitro*. Work on the sites to which the zinc fingers of WT1 bind shows that the molecule recognises the consensus DNA-binding site of the EGR-family of proteins (Rauscher *et al.*, 1990). Such high-affinity binding sites have been identified upstream of *IGF-II* (Drummond *et al.*, 1991; 1992), *Pax-2* (Dressler *et al.*, 1992) and the NGF-receptor gene (Hannu Sariola, personal communication) and may well be present elsewhere. There are, however, two factors that distinguish WT1 from the EGR family: it contains an extra zinc finger, with as yet unknown binding properties, and the most abundant of the four alternative splice variants contains an additional 3 amino acids inserted between the 3rd and 4th fingers, which leads to altered binding specificity (Bickmore *et al.*, 1992). It remains an open question as to whether or not alternative-splice forms of the protein regulate the downstream expression of different sets of genes, although this seems likely with the demonstration that they recognise different binding sites (Rauscher *et al.*, 1990; Bickmore *et al.*, 1992).

In conclusion, our examination of the role of *WT1* in normal kidney development, in the light of the data from the mouse, suggests that each of the three levels of transcription may have a different functional role during the course of nephrogenesis. It is of interest in this context, that the *Drosophila* gap gene *Kruppel* can act as either an activator or a repressor depending on its concentration (Sauer & Jackle, 1991).

## 6.4 The role of *WT1* in tumorigenesis

It is thought that WT arises from the aberrant differentiation of abnormally persistent nephrogenic stem cells. We have seen in the previous section, that based on its expression pattern in the human embryo and its predicted role as a transcription factor, the *WT1* gene product has been proposed as being involved in committing these stem cells to the

nephrogenic pathway, . We can therefore consider the mechanism(s) by which mutation of the *WT1* gene leads to malignancy in the context of its expression and proposed roles in normal kidney development. The proportion of tumours that are associated with *WT1* mutations has been estimated by Southern blot analysis and association with WAGR to be between 5-10%, although this is likely to be a substantial underestimate because many mutations would not be detected with these assays (van Heyningen & Hastie, 1992). Depending on the timing and nature of the mutation, a spectrum of phenotypes could be produced, ranging from congenital abnormalities to malignancy. There is evidence from the associated congenital abnormalities that a mutated protein has a more severe effect on phenotype than if it is absent (Pelletier *et al.,* 1991b,c; Bruening *et al.,* 1992). This, together with the implication of the involvement of two further loci and the variable histological profile of the tumour itself, makes the task of elucidating the relationship between *WT1* mutation and tumorigenesis difficult. While the study presented here can say nothing directly about the effect of mutation, we can use the ideas discussed in the previous section, and in particular the three levels of *WT1* expression, to speculate on how WT could arise as a result of *WT1* aberration.

Previous investigations have shown that the cells of the metanephric mesenchyme will not grow and divide unless they are induced (Grobstein, 1955). It is therefore difficult to envisage how WT, which by its very nature involves uncontrolled cell proliferation, could arise from cells that remain uninduced. This in turn implies that malignancy is unlikely to be a consequence of mutation of the *WT1* locus at the pre-induction stage, even though we have shown that the gene is normally expressed at a low level in uninduced metanephric mesenchyme. It is possible, on the other hand, that mutation or absence of *WT1* at this point in development could lead to renal agenesis, one of the congenital abnormalities associated with the WAGR syndrome (Miller *et al.,* 1964).

An initial response of the mesenchyme to induction is an increase in cell proliferation (Saxen, 1987) and in this study a concurrent increase in the level of *WT1* mRNA was observed. Following induction, the stem cells begin to enter the nephrogenic pathway of

differentiation and *WT1* may play a part in committing these cells. If *WT1* is the critical factor in the switch from proliferation to epithelial differentiation, then its total inactivation would presumably lead to a tumour that was either completely blastemal or that had reverted to a stromal phenotype. This has proven not to be the case, because a triphasic tumour has been shown to be homozygously deleted for the region containing the gene (Lewis *et al.*, 1988). It can be concluded that loss of *WT1* is either not the pivotal factor in such tumours or that there is some means of bypassing it to allow the limited degree of epithelial differentiation seen in this example. If, on the other hand, mutation of *WT1* leads to an altered or even enhanced protein function, then its aberrant expression could lead to the differentiation of epithelial components.

During normal kidney development, a further increase in the level of *WT1* mRNA was observed in the renal vesicle and we suggested that this could be important in regulating the switch to an epithelial phenotype. Mutation or deletion of *WT1* at this stage could be a primary event in tumours with a triphasic morphology.

To summarise, we have shown that *WT1* is expressed at three distinct levels in the developing kidney and suggested that these may indicate different functional roles. This has then been used to explain how this gene could be involved in tumorigenesis at a number of stages during nephrogenesis.

## 6.5 The use of *WT1* as an assay of kidney development

One of the most useful aspects to emerge from this study is that of employing *WT1* as a marker of differentiation in the *in vitro* system of kidney culture. This gene has already been shown to be a practical marker for tumour differentiation, because there is a close correlation in the expression patterns between the malignant tissue and its normal counterpart, with transcription being limited in the tumour to the blastemal cells and immature epithelial components (Pritchard-Jones & Fleming, 1991). During the present study, *WT1* was used as

such a marker, to demonstrate that the growths obtained by the method of Javadpour & Bush (1972) had undergone an inappropriate degree of differentiation for a model of WT.

We have extended this idea to show that *WT1* can be used in much the same way to examine embryonic kidneys that have been experimentally manipulated. From observations made on rudiments cultured under routine conditions, *WT1* was shown to be a good marker of tissue that had been induced and was undergoing the transition to epithelia. It is also the most specific marker of podocyte cells that has been described so far and may be of benefit in future *in vitro* studies, because the formation of Bowman's capsule is often used to determine the degree of differentiation achieved during an experiment.

In addition, *WT1* was employed as a marker in rudiments that had been cultured in medium containing either LIF or cytochalasin B, substances that had previously been shown to block nephrogenesis (Bard & Ross, 1991; Bard, 1990a). This served two purposes, it allowed the further investigate of these molecules and in doing so illustrated the application of this gene in future studies. By examining the expression pattern of *WT1,* it was possible to determine that both molecules acted at a point downstream of induction, but prior to the transition of the mesenchymal aggregate to epithelium. Furthermore, the data confirmed the observation by Bard & Ross (1991) that LIF blocks nephrogenesis at a second stage, arresting the further development of renal aggregates that had undergone epithelialisation.

## 6.6 The limitations of using the mouse to study *WT1* and Wilms' tumour

Despite the almost identical patterns of expression of the *WT1* gene in the human and mouse embryonic kidneys, we cannot escape the fact that the latter do not develop WT. It has been suggested that this could reflect differences in the genetic complexity between these species: the two loci on the short arm of chromosome 11 in the human are on separate chromosomes in the mouse, so that the homologous region of 11p13 is on chromosome 2 and that of 11p15 on chromosome 7. Another hypothesis is that the

population of target cells is smaller or more short-lived in the mouse (Glaser *et al.*, 1990), although the demonstration here, that *WT1* is still being transcribed shortly before birth in a few cells in the outer cortex of the kidney, suggests that the stem cells are more persistent in this species.

We must therefore consider briefly how appropriate it is to study *WT1* expression in the mouse. The benefits of using this species have already been laid out in previous chapters and coupled to the similarity in the expression patterns between the two species, indicate that the mouse is of value in investigating the normal function of this gene.

It had been hoped that by studying a system that had been proposed to produce WT in mice (Javadpour & Bush, 1972), further insight could be gained into the early stages of the eponymous tumour. The demonstration that it was neither an accurate or reliable model of the malignancy prevented this aim from being pursued further.

## 6.7 Future directions

In conclusion, it seems that the *in situ* mRNA hybridisation analysis raises more questions than it answers and it is clear that those questions which centre around the functional roles of *WT1* need to be examined by direct experimentation. This can be approached in two distinct ways, by culturing mouse kidney rudiments in the presence of anti-sense thio-oligonucleotides which will block *WT1* expression (e.g. Sariola *et al.*, 1991) and by making transgenic mouse with an incapacitated *WT1* gene. Together, the results should illuminate the role of *WT1* in development and may lead to a better understanding of the molecular basis of kidney formation.

In a wider context, the study of the alternative splice forms of WT1 may help to clarify the more diverse aspects of the expression pattern described here. In the long-term, the isolation and characterisation of the genes from the other loci implicated in the formation of WT will be of paramount importance. It seems likely that these will turn out to either

regulate *WT1*, or be its downstream targets and the *in vitro* system of kidney culture should allow the temporal and spatial relationship of these genes to be investigated. The results presented in this thesis provide a framework for such studies in the future.

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# APPENDIX

The complete nucleotide (3,089 bp) and predicted amino acid sequence of the mouse WT1 cDNA. The region of the polypeptide spanning the zinc-finger domain is in bold (Buckler et *al., 1991*). The cDNA used to transcribe the riboprobe employed in this thesis was 2,362 bp in length and started at the nucleotide marked.

```
tgtgtgaatggagcggccgagcatcctggctcctcctccttccctgctgccggcccctcttatttgagctttgggaagctg
ggggcagccaggcagctggggtaaggagttcaaggcagcgcccacacccggggctctccgcaacccgaccgcctgcctgcc
tccccctttccttttttccccgcccctcctcccacccactcattcacccacccacccagagagaggacggcagcccagga
acccgggcccgccgcctcctcgccgcgatcctggacttcctcctgtcgcaggagccggcttccacgtgtgtcccggagccg
gcgtctcagcacacgctccgccgggagcccgggtgcgtccagcagccggagcaacctggggaccgaggcccccggagcgcc
                                                                    M  G  S
tgggccaagtccagcgccgagaatccgcaggatcgcaggagcggagaaccgtccgcatccgagccgcacctcatgggttcc
 D  V  R  D  L  N  A  L  L  P  A  V  S  S  L  G  G  G  G  G  G  C  G  L  P  V  S
gacgtgcgggacctgaacgcgctgctgcccgctgtgtcttcgctgggcggcggcggcggcggctgcgggctccctgtgagc
 G  A  R  Q  W  A  P  V  L  D  F  A  P  P  G  A  S  A  Y  G  S  L  G  G  P  A  P
ggcgcacggcagtgggcgcccgtgttggacttcgcgcctccgggcgcctcggcttacgggtcgctgggcggtcccgcgcct
 P  P  A  P  P  P  P  P  P  P  P  H  S  F  I  K  Q  E  P  S  W  G  G  A  E  P  H
cctcccgctccgccgccgcctccgccgccaccccactccttcatcaaacaggagcccagctggggcggcgccgagccacac
 E  E  Q  C  L  S  A  F  T  L  H  F  S  G  Q  F  T  G  T  A  G  A  C  R  Y  G  P
gaggagcagtgcctgagcgccttcaccttgcacttctcgggccagttcaccggtacagccggggcctgtcgctacggaccc
 F  G  P  P  P  P  S  Q  A  S  S  G  Q  A  R  M  F  P  N  A  P  Y  L  P  S  C  L
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catcacgcggcgcagttccccaaccattccttcaaacacgaggaccccatgggccagcagggctcgctgggcgagcagcag
 Y  S  V  P  P  P  V  Y  G  C  H  T  P  T  D  S  C  T  G  S  Q  A  L  L  L  R  T
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 P  Y  S  S  D  N  L  Y  Q  M  T  S  Q  L  E  C  M  T  W  N  Q  M  N  L  G  A  T
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 L  K  G  M  A  A  G  S  S  S  S  V  K  W  T  E  G  Q  S  N  H  G  T  G  Y  E  S
ttaaagggaatggctgctgggagctccagctcagtgaaatggacagaagggcagagcaaccacggcacagggtacgagagt
 E  N  H  T  A  P  I  L  C  G  A  Q  Y  R  I  H  T  H  G  V  F  R  G  I  Q  D  V
gagaaccacacggcccccatcctctgtggtgcccagtacagaatacacacccacggggtcttccgaggcattcaggatgtg
 R  R  V  S  G  V  A  P  T  L  V  R  S  A  S  E  T  S  E  K  R  P  F  M  C  A  Y
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 P  G  C  N  K  R  Y  F  K  L  S  H  L  Q  M  H  S  R  K  H  T  G  E  K  P  Y  Q
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 C  D  F  K  D  C  E  R  R  F  S  R  S  D  Q  L  K  R  H  Q  R  R  H  T  G  V  K
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ccattccagtgtaaaacttgtcagcgaaagttttcccggtccgaccatctgaagacccacaccaggactcatacaggtaaa
 T  S  E  K  P  F  S  C  R  W  H  S  C  Q  K  K  F  A  R  S  D  E  L  V  R  H  H
acaagtgaaaagcccttcagctgtcggtggcacagttgtcagaaaaagtttgcgcgctcagacgaattggtccgccatcac
 N  M  H  Q  R  N  M  T  K  L  H  V  A  L
aacatgcatcagagaaacatgaccaaactccacgtggcgctttttcccggtccgaccatctgaagacccacaccaggact
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gcatcccaggcaggaaagtgtgcaaactgcttccaaatctgattttgaaattcctcccactcacctttcaaaggacacgac
```

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tactgtagcttgaaatactgtgtaaatatatatgtatatatatgatgtgctctttgtcaactaaaattaggaggtgtatg
gtattagctgcatcactgtgtggatgtcaatcttacagtgtattgatgataatactaaaaatgtaacctgcatctttttc
cacttggctgtcaattaaagtctattcaaaagga

Comparison of the predicted amino acid sequences of the mouse and human WT1 polypeptide. Vertical bars indicate amino acids that are conserved between the two species and the alternative splices are in bold (Buckler *et al.*, 1991).

```
mouse    MGSDVRDLNALLPAVSSLGGGGGGCGLPVSGARQWAPVLDFAPPGASAYG      50
         ||||||||||||||| || |||||| |||||| ||||||||||||||||
human    MGSDVRDLNALLPAVPSL-GGGGGCALPVSGAAQWAPVLDFAPPGASAYG      49

         SLGGPAPPPA-PPPPPPPHSFIKQEPSWGGAEPHEEQCLSAFTLHFSGQF      100
         |||||||||| |||||||||||||||||||||||||||||||| ||||||
         SLGGPAPPPAPPPPPPPPHSFIKQEPSWGGAEPHEEQCLSAFTVHFSGQF      100

         TGTAGACRYGPFGPPPPSQASSGQARMFPNAPYLPSCLESQPTIRNQGYS      150
         |||||||||||||||||||||||||||||||||||||||| |||||||
         TGTAGACRYGPFGPPPPSQASSGQARMFPNAPYLPSCLESQPAIRNQGYS      150

         TVTFDGAPSYGHTPSHHAAQFPNHSFKHEDPMGQQGSLGEQQYSVPPPVY      200
         |||||| ||||||||||||||||||||||||||||||||||||||||||
         TVTFDGTPSYGHTPSHHAAQFPNHSFKHEDPMGQQGSLGEQQYSVPPPVY      200

         GCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQLECMTWNQMNLGATLKGM      250
         ||||||||||||||||||||||||||||||||||||||||||||||||
         GCHTPTDSCTGSQALLLRTPYSSDNLYQMTSQLECMTWNQMNLGATLKGV      250

         AAGSSSSVKWTEGQSNHGIGYESENHTAPILCGAQYRIHTHGVFRGIQDV      300
         ||||||||||||||||  |||| ||| ||||||||||||||||||||||
         AAGSSSSVKWTEGQSNHSTGYESDNHTTPILCGAQYRIHTHGVFRGIQDV      300

         RRVSGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGE      350
         ||| ||||||||||||||||||||||||||||||||||||||||||||||
         RRVPGVAPTLVRSASETSEKRPFMCAYPGCNKRYFKLSHLQMHSRKHTGE      350

         KPYQCDFKDCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKT      400
         ||||||||||||||||||||||||||||||||||||||||||||||||||
         KPYQCDFKDCERRFSRSDQLKRHQRRHTGVKPFQCKTCQRKFSRSDHLKT      400

         HTRTHTGKTSEKPFSCRWHSCQKKFARSDELVRHHNMHQRNMTKLHVAL      449
         |||||||||||||||||| |||||||||||||||||||||||  ||
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```